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Evidence suggests that nutrients in donor human milk (DHM) are profoundly variable, yet there has been little exploration into how processes within milk banking affect nutrient variability. We hypothesized that processing methods for thawing, decanting, pooling, and mixing during bottling contribute to nutrient retention and distribution in DHM. Our investigation — based on observations from an environmental scan of 9 milk banks in the Human Milk Banking Association of North America (HMBANA) network — was divided into three specific aims.

Aim 1: Two pooling attributes — number of donors per pool and if macronutrient analysis was used to select donors for a pool ("target pooling"; yes/no) — were assessed for fat and protein variability using samples of raw, pooled DHM from 20 milk banks (n = 300). Target pooled samples had less fat variability ($p = 0.04$). In samples not target pooled, more donors per pool reduced fat and protein variability ($p < 0.05$). Aim 2: Two thaw stages (ice/liquid) and the use of bag manipulation during decanting (yes/no) were assessed for fat retention and bacteria colony forming units. Fresh milk samples (n = 40) were divided into storage bags and frozen at -20ºC for 2 months. Decanting with bag manipulation retained more fat than decanting without bag manipulation, but only when milk was thawed to a liquid state ($p = 0.005$), not an ice state ($p = 0.47$). Bag manipulation did not increase bacteria ($p = 0.49$). Aim 3: Six mixing during bottling treatments were tested using pools of raw DHM — pooling container material (plastic/glass), duration of pre-bottling refrigerated storage (1-hour/24-hours), mixing during bottling method (manual/mechanical; one no-mixing group). Pooled DHM was mixed using the assigned treatment, bottled, then measured for fat, protein, IgA, and lysozyme ($n = 6$ pools and 114 samples). Holding a pool in the refrigerator 24-hours before bottling created greater fat variability compared to 1-hour ($p < 0.01$). No differences in nutrient variability were observed between glass/plastic containers or manual/mechanical mixing methods ($p > 0.05$).

In conclusion, fat content was affected by several steps in DHM processing, but the impact may be mitigated under certain conditions: When processing milk frozen in a plastic storage bag, fat retention may be improved by using bag manipulation during decanting when thawing to a liquid state. When milk is pooled on one day and bottled on a subsequent day, more mixing is needed to reduce fat variability. Additionally, for milk banks that do not target pool, using a greater number of donors per pool may reduce both fat and protein variability.

THE IMPACT OF PROCESSING ON THE RETENTION AND DISTRIBUTION OF MACRONUTRIENTS AND BIOACTIVE COMPOUNDS IN DONOR HUMAN MILK

by

Linda Lee Friend

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CHAPTER I

INTRODUCTION

The rate of preterm births (< 37 weeks gestation) in the United States has been on the rise since 2015, and accounts for nearly 10% of live births.[1] Preterm infants are 9-times more likely to be admitted to the neonatal intensive care unit (NICU),[2] and have an increased risk of morbidity and mortality, as well as many other negative short– and long–term health outcomes, including both cognitive and physical delays and/or disabilities.[3,4] Many of these issues are nutritionally driven — the immature gastrointestinal tract cannot adequately digest, absorb, or metabolize nutrients, leading to nutrient deficiencies and energy intakes that are insufficient to support growth.[5–7] Growth failure in preterm infants is a systemic problem,[8] with almost 50% of preterm NICU infants assessed as below the 10th percentile on growth charts at discharge.[9–11]

The specific nutrient needs of a preterm infant remains an area of contention, but it is known that low intakes of energy and protein can negatively impact growth.[12–16] Feeding protocols vary by NICU, and incongruent practices have been implicated in the high rate of growth restriction,[11,17,18] with many NICUs regularly failing to meet minimum intake recommendations.[11,19] There is, however, a commonality between feeding protocols — the prioritization of human milk (HM). Experts — such as the American Academy of Pediatrics, Academy of Nutrition and Dietetics, and World Health Organization — strongly support the use of HM; and if mother's own milk (MOM) is unavailable, donor human milk (DHM) is recommended before infant formula.[20–24] Evidence suggests that nutrients in DHM are highly variable, especially fat.[25–32] For reference, 1 g/dL of fat translates to 2.7 kcal/oz, thus swings in fat have a significant impact on calories.

Preterm infants fed DHM have inferior growth compared to those receiving formula or MOM,[33–36] which may be associated with nutrient levels in DHM that do not align with the expected values used in many NICU feeding protocols.[13–15] Despite inferior growth,[37–39] governing bodies still recommend DHM over formula, largely due to a lower risk of necrotizing enterocolitis (NEC),[40] a life-threatening condition that affects 15% of NICU infants.[41] While much is still unknown about the etiology of NEC, there is substantial evidence that preterm infants fed DHM are at a lower risk than those fed formula.[35,40,42–44] Some evidence suggests that bioactive compounds, such as immunoglobulin A (IgA) and lysozyme, contribute to a lower risk of NEC by providing early and important immune protection against pathogens in the intestinal tract.[45–47]

The use of DHM in the NICU is increasing, with a 74% increase between 2011 and 2015.[48] NICUs receive DHM from milk banks, where milk undergoes a variety of production processes. Typical steps include donor screening, receiving and storing frozen milk, thawing, decanting, pooling, mixing, bottling, and pasteurizing.[49] The impact of pasteurization on nutrients in DHM has been vastly studied,[50] but there has been little exploration into other processing steps within a milk bank that may influence nutrients. For example: Research about thawing HM typically uses small volumes of HM or methods not realistic at a milk bank scale (e.g. microwaves).[51–54] Studies related to decanting only remove partial volumes of HM (versus completely emptying the container), and few use storage bags (which are common in milk banks).[55,56] There is evidence that pooling multiple donors can decrease nutrient variation,[12,27,31] and some milk banks choose to combine donors based on the results of macronutrient analysis, but it is not required. Research about mixing has largely been based on studies from the dairy industry, or using small volumes of HM in a clinical setting to improve fat delivery during gastric tube feedings.[57,58]

The purpose of this research was to examine how previously unexplored steps in DHM processing may affect the variability of macronutrients and bioactive compounds in raw DHM. We hypothesized that processing methods for thawing, decanting, pooling, and mixing during bottling would influence nutrient variability in DHM. The investigation was divided by processing methods into three specific aims: Aim 1: Describe the variability of fat and protein in a large set of DHM samples from multiple milk banks, and assess potential relationships with two processing factors — pre-pooling macronutrient analysis and the number of donors in a pool. Aim 2: Determine how thaw stage and method for decanting HM from plastic storage bags influences the retention of fat and bacteria. Aim 3: Determine how different methods of mixing during bottling impact the distribution of macronutrients and bioactive factors in bottled, raw DHM.

Overall, the goal of this research is to inform evidence-based guidelines for milk banking by identifying associations between DHM processing methods and nutrient variability; and evaluating the impact of those processes on the retention and distribution of fat, protein, IgA, and lysozyme in raw DHM. Moreover, the major long-term goal of this research is producing DHM with a more consistent nutrient profile to improve growth outcomes for preterm infants.

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CHAPTER II

REVIEW OF THE LITERATURE

Impact of Human Milk Macronutrients on Preterm Infant Growth

Human milk (HM) is an intricate matrix of nutrients and bioactive factors, and this review focuses on macronutrients (fat, carbohydrate, and protein), which are known to be highly variable — between individuals, throughout the day, and from day-to-day.[1–9] Despite this knowledge, the use of assumed and constant HM macronutrient values is a major weakness that plagues preterm infant growth studies. Without measuring macronutrients, it cannot be assured that infants are receiving adequate nutrition, and inferring a causal dose-response relationship between HM and growth restriction is spurious at best.[10] This section provides an overview of the relationships between HM macronutrients and growth outcomes in the preterm infant, with a focus on studies that measured macronutrients.

A clinical trial by Rochow et al[11] compared the impact of two HM fortification protocols — standard fortification (SF; unknown/assumed nutrient content) and target fortification (TF; known/measured nutrient content) — on weight gain in preterm infants (mean 26 weeks gestation and 860 g birth weight) on an exclusive HM diet. For 3 weeks, infants in the intervention group (n = 10) were fed using a 3-step TF: First, macronutrients were measured in unfortified HM using a human milk analyzer (HMA); next the HM was fortified per SF and measured again; then additional macronutrients were added to meet guidelines published by the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN).[12] HMA results were adjusted per an internal validation using chemical analysis. The authors pointed out that all HM (n = 650 samples) required the full 3-step TF to reach ESPGHAN recommendations — after SF (step 2), none of the samples reached minimums for protein and carbohydrate (CHO), and only 55% reached the minimum for fat. Infants in the intervention group were matched with two other similar infants from a historical control group fed HM with SF (n = 20). Although there were no significant differences in mean weight gain (about 20 g/kg/d for both groups), there was a significant correlation between HM intake and weight gain in the intervention group ($R^2 = 0.68$, $p = 0.004$), but not in the control ($R^2 = 0.02$, $p = 0.58$). The data showed that accounting for macronutrient variation through TF resulted in predictable growth patterns, while the unknown macronutrient content in SF resulted in random growth patterns. The process of SF does not take into account the wide range of macronutrients in HM, hence infants in the control group received wide ranges of nutrition. Interestingly, the intervention group was supposed to receive the same feeding volume as the control group, but instead received an average of 8.4 mL/kg/d less, due to hospital staff providing less HM because of concerns over higher-than-expected weight gain. The researchers speculated that weight gain in the intervention group would have been higher, had the hospital staff followed study protocol.[11]

In addition to body weight, preterm infant growth outcomes are commonly expressed using body length and/or head circumference (HC),[13] and some experts argue that body composition should also be included.[14] Research suggests that the type of weight gain is important — increases in adiposity (percent of body weight as fat mass, %FM) may increase the risk of both childhood and adult obesity,[15,16] while increases in fat free mass (%FFM) are associated with better neurodevelopmental outcomes (per Bayley Scales of Infant Development scores).[17] Multiple growth outcome measurements were assessed in an observational study by Piemontese et al,[18] which investigated the relationship between nutrition source (percent of diet from HM or formula) and growth outcomes. HM was from mother's own milk (MOM) and/or donor human milk (DHM). Preterm infants ($n = 73$, mean 30 weeks gestation and 1248 g birth weight) were stratified by percentage of diet from HM, with one group consuming < 50% HM ("mostly formula") and the other group consuming ≥ 50% HM ("mostly HM"). Actual percent of HM in the diet was significantly different between groups ($p < 0.001$), where the mostly formula group ($n = 24$) consumed 35% HM (of which 42% was MOM) and the mostly HM group ($n = 49$) consumed 81% HM (of which 76% was MOM). An HMA was used to measure HM macronutrients for TF. Mean daily intakes of energy and protein were not significantly different between the groups (averages at discharge = 131 kcal/kg/d and 3.6 g/kg/d protein). Despite similar nutritional intakes, mean daily growth rate was 1.8 g/kg/d higher in the group receiving mostly formula ($p = 0.002$), yet both groups achieved the goal growth rate of ≥ 15g/kg/d, and there were no significant differences in weight, length, HC, or %FFM between the groups at discharge (study duration approximately 52 days). A multiple regression analysis indicates a positive association ($\beta = 0.12$, $p = 0.01$) between a diet consisting of ≥ 50% HM (MOM and/or DHM) and improved body composition (%FFM). Other studies have also reported higher rates of %FFM accretion in infants fed HM compared to those fed formula, particularly when using TF to control for variations in HM macronutrients.[15,16] A study from de Halleux et al[19] suggests TF may provide consistent nutrient intakes that result in predictable growth outcomes in preterm infants exclusively fed HM (MOM and/or DHM). McLeod et al[20] identified a relationship between protein and weight gain, and found for each additional g/kg/d of protein, weight gain velocity (g/kg/d) increased by 9% ($p = 0.024$); and protein intakes above 3.4 g/kg/d reduced gains in adiposity (%FM) by 2% ($p = 0.042$).[20]

A deeper probe into the relationship between nutrient intake and multiple growth outcomes was conducted by Stoltz Sjostrom et al.[21] This study investigated growth (weight, length, and HC) in Swedish preterm infants (< 27 weeks gestation) during the first 70 days of life. Infants (n = 394) were fed fortified MOM and DHM, but this was not stratified in the analysis. Macronutrient content was measured using an HMA for 68% of the participants (assumed averages were used for the remaining 32%). Although the reported mean daily energy intake of 120 kcal/kg is within published guidelines,[12,22,23] most of the infants were below energy recommendations during the first month of life, with a mean daily intake of 66 kcal/kg during this time. Similarly, protein intake was insufficient during the entire study period, with an overall mean daily intake of 3.2 g/kg, which is lower than the recommendations used by the hospitals in the study.[23] Fat and CHO intake were approximately within recommendations. Overall growth (weight, length, and HC) had a positive linear relationship with energy intake, protein intake, and percent of calories from protein, but energy intake was a better predictor of overall growth. Looking at the growth outcomes individually, energy intake was also the strongest predictor of improvements in weight ($\beta = 0.315$, $p < 0.001$), and percent of calories from fat was an independent predictor of HC ($\beta = 0.146$, $p = 0.018$), but no associations could be made about gains in length, which were seemingly less reactive to nutrient intakes.[21] Similar results were found by Asbury et al,[24] who conducted a retrospective analysis of data from a randomized control trial called Donor Milk for Improved Neurodevelopmental Outcomes (GTA-DoMINO). Weight gain was associated with average daily intakes of energy and all three macronutrients. Associations between gains in length and average daily intakes of energy and macronutrients were only seen during the first 8 days of life, when infants were still receiving parenteral nutrition. HC increase was associated with average daily intakes of energy, fat, and protein. Although macronutrients were not measured, these conclusions may still be appropriate because the SF protocol used different assumed values for HM macronutrient content over the course of the study, in order to simulate changes in HM composition seen throughout lactation.[24]

These collective results underscore the clinical significance of HM macronutrient content and suggest that low energy and protein concentrations may have negative consequences on preterm infant growth. Evidence reveals that preterm infants fed DHM have inferior growth compared to those fed formula or MOM.[10,19,25,26] Target fortification has been shown to be beneficial for preterm infants,[11] but is also more labor-intensive than other fortification methods and may not be feasible in some neonatal intensive care units (NICU).[20]

Macronutrient Composition of Donor Human Milk

Current evidence suggests that while preterm infants fed DHM have significantly lower rates of necrotizing enterocolitis than those fed preterm formula, they also have inferior in-hospital growth.[26] This section will address what is known regarding the macronutrient composition of DHM. In order to provide an accurate representation of DHM used in NICUs in the United States (US), the following criteria were selected to identify studies: the term "DHM" was used to describe a collection of pooled HM; DHM samples were obtained from a milk bank in the Human Milk Banking Association of North America (HMBANA) network and/or DHM was created per HMBANA guidelines[27]; research was conducted in North America; and method of macronutrient measurement was described.

Initial search results indicated that the term "pool" has been used to describe a collection of HM from a single donor or from multiple donors, but HMBANA guidelines specify that a pool contains milk from "more than one donor."[27] Several studies used "pool" to refer to HM collected by a single donor over a 24-hour period, but this is not representative of HM donated to a milk bank, which is typically single donations that have been collected over weeks or months.[28] For these reasons, studies using 24-hour single-donor pools were excluded. Similarly, milk bank samples from specialty pools (e.g. outpatient/not intended for NICU use, skim, "dairy-free") were also excluded. North America (US and Canada) was used because several milk banks in the HMBANA network are located in Canada, but studies from other regions were excluded due to different practices regarding donation and handling of DHM (e.g. a study from Brazil accepted HM from donors as young as 15 years old,[29] Swedish donors rarely donate after 3 months post-partum,[30] and DHM in the United Kingdom was treated with an antimicrobial agent in lieu of pasteurization[31]). Pasteurization status was not included because an extensive review indicates that energy and macronutrient contents of Holder-pasteurized DHM are mostly retained.[32]

The final search yielded only eight studies that matched the criteria, which are summarized in Table 1. Some studies reported DHM nutrients both pre– and post–pasteurization, but only post-pasteurization measurements are included in the table below since all HMBANA banks pasteurize DHM intended for NICU use.[27]

Strengths and Weaknesses of Analytical Methods

With a combined total of 1,414 samples from multiple locations across the US and Canada, the DHM studies outlined in Table 1 provide insight about the macronutrient composition of DHM used to feed preterm NICU infants. However, one study accounts for 79% of the samples (1,111/1,414), and four studies have a sample size under 30. Additionally, there were varying degrees of detail about methodology — for example, Ley et al[33] provided a general outline while Perrin et al[34] specified intricate details. Half of the studies were strengthened by the use of replicate measurements with low coefficients of variation (CVs; reported values ranged 1.6–6.8%).[35] Of the studies that provided information about aliquoting and sample preparation, appropriate methods for obtaining representative samples (e.g. adequate mixing)[36–38] were used in all but one study — Fu et al[39] saved samples of "remaining milk" that were left over after DHM had been removed from the bottle and used to create a fortified feed. If only a small volume

Table 1. Macronutrient Composition of Pooled Donor Milk in North America.

Author (Year)	**n**	**Energy (kcal/dL)**	**Fat (g/dL)**	**CHO/Lactose (g/dL)**	**Protein (g/dL)**	**Method**	**DHM Sample Characteristics**
Ley[33] (2011)	17	69.4±8.8	3.9±0.8	NA	1.5±0.1	Bomb calorimetry, CMCT, BCA	Pasteurized; created pools
Donovan[41] (2017)	21	63.9	3.7±0.4	6.7±0.2	1.1±0.1	HMA (LactoScope)	Pasteurized; from milk bank
Meredith-Dennis[42] (2017)	3	63.6±8.3 (57.3–72.9)	3.4±0.9 (2.8–4.6)	7.2±0.01 (7.2–7.3)	1.0±0.1 (0.9–1.1)	HMA (LactoScope)	Pasteurized; from milk bank
Perrin[34] (2017)	33	NA	3.5±1.7	*Lactose* 5.6±0.7	1.5±0.2	SMART Trac, LC-MS, BCA	Raw; from milk bank
Castro[43] (2019)	10	61.5	3.1	7.0	1.0	HMA (Miris)	Pasteurized; created pools
Fu[39] (2019)	96	62.3±5.8 (41.4–74.2)	3.1±0.6 (1.5–4.5)	7.7±0.4 (6.3–8.5)	0.9±0.2 (0.3–1.4)	HMA (SpectraStar)	Pasteurized; from milk bank
John[44] (2019)	1,111	NA	(2.7–5.9)	NA	(0.8–2.2)	HMA (MilkOScan)	Raw; from milk bank
Young[45] (2019)	123	58.6±5.7 (49.0–76.9)	2.9±0.6	7.0±0.2	0.7±0.1	HMA (Miris)	Pasteurized; from milk bank

Note: Values, when applicable, are listed as: mean ± standard deviation (minimum – maximum). DHM — donor human milk; CHO — total carbohydrate; NA — not applicable/values not reported; CMCT — creamatocrit method for measuring fat; BCA — bicinchoninic acid assay for measuring protein; HMA — human milk analyzer that uses infrared technology to determine macronutrient content; SMART Trac — device used to quickly measure moisture and fat content using nuclear magnetic resonance; LC-MS — method for measuring lactose via graphitic carbon high-performance liquid chromatography with tandem mass spectrometry.

of leftover milk remained, the high ratio of surface area may provide more opportunities for fat to cling to the bottle, which translates to artificially lower fat (and thus energy) content.[37,40] Interestingly, the lowest reported values for fat and energy were from Fu et al.[39]

Several methods for measuring HM macronutrients have been validated against established chemical reference methods,[46] such as the Mojonnier[47] ether extraction or the Rose-Gottlieb method for fat; and Kjeldahl[48] nitrogen analysis for crude protein, non-protein nitrogen, and true protein.[49] There is a lack of agreement concerning a standard method for quantifying and reporting CHO in HM, although many studies measure only lactose, not total CHO (which includes indigestible sugars that do not significantly contribute to total energy[50]).[40,46,49,51] For example, graphitic carbon high-performance liquid chromatography plus mass spectrometry (LC-MS) was shown to be accurate and precise for lactose.[49,52] LC-MS was used by Perrin et al,[34] and was the only study in Table 1 to measure lactose, hence the lower values in comparison to the other DHM studies (that measured total CHO).[39,41–43,45]

Findings from Studies Using Human Milk Analyzers

Six of the eight DHM studies used human milk analyzers (HMA) to measure macronutrients. Several brands of HMAs are commercially available, but only the Miris HMA (used in two DHM studies) was FDA-approved as a medical device.[53] An overview of several validation studies show conflicting results regarding accuracy and precision of HMAs, especially with regard to lactose.[40] The MilkOScan (used in the study by John et al[44]) had good accuracy when calibrated for HM, including high precision (CV < 2%) and high correlation with references for protein and fat (slope = 1), but poor correlation with lactose (slope <> 1).[54] The Miris HMA (used by Castro et al[43] and Young et al[45]) was accurate and reliable for fat and lactose, but not protein, with average measurements 0.48 g/dL lower than the Kjeldahl method ($p < 0.05$). It is important to note that HMAs measure total CHO,[55] and a correction factor should be used to calculate lactose content prior to comparing HMA results with reference methods that measure lactose.[56] Unfortunately, some authors have used CHO and lactose as interchangeable terms with no mention of a conversion, which may be contributing to conflicting results.[41,57] A more recent investigation, however, demonstrated that a variety of HMAs were reliable and accurate for measuring fat and protein when used in a milk bank setting.[58]

HMAs measure macronutrients via infrared technology — where a volume of HM (ranging 1.5–45 mL) is exposed to infrared radiation, and wavebands indicate chemical structure vibrations that are unique to each macromolecule.[59] However, nitrogen bonds are used to determine crude protein content, which includes non-protein nitrogen-containing molecules (e.g. urea), and a correction factor (reported values between 20–50%[58]) should be used to estimate the "true" protein content.[56] Of the six DHM studies that used HMAs, three studies did not differentiate between crude or true protein in their results. Crude protein was reported in the studies from Meredith-Dennis et al[42] and Castro et al[43]; both averaged 1.0 g/dL, although samples sizes were very small (n = 3 and 10, respectively). True protein was reported by Young et al,[45] which may provide explanation for the average protein content of 0.7 g/dL, which was the lowest overall (despite being the second largest study).

Findings from Studies Not Using Human Milk Analyzers

Protein: In the two DHM studies that did not use an HMA, protein content was determined by the Pierce bicinchoninic acid (BCA) assay. This method has been validated to measure protein in HM ($R^2 = 0.99$),[60] although it likely overestimates by as much as 30%.[46] BCA is based on the reduction of Cu^{+2} to Cu^{+1} via biuret reaction, and Cu^{+1} is colorimetrically detected via spectrophotometer. The purple-colored product has a strong absorbance at 562 nm, the intensity of which is proportional to protein concentrations ranging 20–2000 µg/mL.[61] Protein content (g/dL) is quantified with a standard curve, but the value should be adjusted using the formula established by Keller and Neville.[60] The two DHM studies that measured protein using this method do not indicate that BCA values were adjusted.[33,34] Average protein content, 1.5 g/dL, was the same in both studies, and the highest reported protein content overall.[33,34] The adjusted value equates to 1.0 g/dL, which is much closer to protein contents reported in the other DHM studies.

Fat: Creamatocrit was used to measure fat in one of the DHM studies.[33] This method, determined by Lucas et al,[62] uses the percentage of cream (expressed as CMCT%, sometimes abbreviated to CMCT) to calculate the fat content of HM (expressed as %fat and g/L or equivalent conversion). It is quick, inexpensive, and does not require a large volume of HM, but the measurement is more operator-dependent than other tests.[46,63] Despite the subjectivity, Meier et al[64] found creamatocrit to have high intra– and inter–user reliability (all mean differences < 1%), and ability to measure fat content similarly to other common laboratory procedures. In a comparison of three methods for measuring fat, Du et al[65] found creamatocrit to be precise (CV = 3.9%) and have a close correlation ($R^2 = 0.995$) with the gravimetric method (a gold standard reference method), despite under-reporting by 0.3–0.6 g/dL. On the other hand, O'Neill et al[66] concluded that the creamatocrit method overestimated fat (and thus energy), compared to an HMA and controls. However, the terms "%fat" and "CMCT" are used interchangeably throughout the paper, which is incorrect. The methods validated by Meier et al[64] established a conversion formula to transform CMCT to g/dL of fat, which was not used by O'Neill. If the reported CMCT measurements (mean = 4.7 CMCT%) for the control were converted to fat using Meier's formula, the values (mean = 3.18 g/dL) would be much closer to the control (mean = 3.22 g/dL) and measurements from the HMA (mean = 3.2 g/dL), and likely would have led to a different conclusion by the authors.

Energy: Bomb calorimetry has been used as the reference method for total energy,[46] but this method is not common because it requires a large volume of HM and specialized equipment (that is no longer commonly produced[35]). Ley et al[33] was the only DHM study to measure energy, and average content (69.4 kcal/dL) was the highest of the DHM studies. Conversely, this is in opposition to the findings of a systematic review and meta-analysis, which concluded that measured energy content of term HM using bomb calorimetry was as much as 6 kcal/dL lower, compared to calculated energy values based on individual macronutrient measurements ($p < 0.001$).[2] While the authors speculate these differences may be attributed to using uncorrected protein and/or CHO values from

HMA measurements, drawing conclusions from a cross-sectional comparison may be specious given the wide reported nutrient variability in HM.[2]

Table 2 summarizes the findings from the studies in Table 1, and compares them to other published values for HM composition — including a recent review of HM composition in the United States published by the USDA,[36] and two examples of clinical reference (assumed) values.[67,68] CHO content was not included for the following reasons: lack of agreement concerning a standard reference measurement,[51] lack of consistent measurement units (total CHO vs lactose only),[46] and conflicting validity of values obtained via HMA.[40]

Table 2. Comparison of Reported and Reference Values for Energy, Fat, and Protein in Human Milk.

	Energy (kcal/dL)	**Fat (g/dL)**	**Protein (g/dL)**
Reported Values			
Literature Search of DHM Composition[33,34,39,41–45] (Table 1)	(41.4–76.9)	(1.5–4.6)	(0.3–1.5)
USDA Review of HM Composition[36]	(54.2–73.1)	(2.9–4.7)	(0.8–1.6)
Reference Values			
Academy of Nutrition and Dietetics[67]	65	3.2	1.2
Baylor College of Medicine[68]	68	3.5	0.9

Note: Values are listed as mean or (minimum – maximum). HM — human milk; DHM — donor human milk; USDA — United States Department of Agriculture.

Table 2 highlights the discrepancy between the reported (actual) nutrient content and the reference (assumed) nutrient content. A major problem is that the reference values used in standard fortification protocols do not account for the wide distribution of nutrients in DHM. To put this into perspective, a retrospective study by Newkirk et al[69] compared assumed vs actual intakes of calories and protein for 29 preterm infants who received DHM fortified according to SF protocol. Macronutrients in DHM were measured daily (via HMA), but those values were only used for post-study analysis. Complete data were available for 78 feedings of DHM, which contained an average of 60.6 kcal/dL (ranged 49.3–76.3 kcal/dL) and 1.1 g/dL protein (ranged 0.9–1.6 g/dL). For reference, the average reported calorie content was 6.1 kcal/dL lower than the reference value per SF protocol. Calorie and protein intakes were calculated using the recorded intake volume and nutrient content of the fortifier used for SF. Mean daily intakes for calories and protein fell within ESPGHAN guidelines. However, looking at the data on a daily basis, minimum calorie requirements were not met 59% of the time, and minimum protein requirements were not met 10% of the time.

Collectively, the data justify the need for strategies to reduce DHM variability. Whether milk banking processes contribute to the observed variability in DHM nutrients is an important to topic to consider.

Impact of Milk Banking Processes on Macronutrients in Donor Human Milk

This section provides background information on milk banking, including the identification of processing methods and how they may impact the macronutrient content of DHM.

Overview of DHM Production

The Human Milk Banking Association of North America (HMBANA) is a non-profit organization established in 1985 whose mission is to "ensure an ethically sourced and equitably distributed supply of donor human milk."[27] They publish evidence-based guidelines for milk banking processes to ensure product safety, which are regularly updated based on current scientific knowledge.[27] The guidelines cover how to establish and operate a milk bank, as well as standards for screening, processing, and distributing DHM. There are currently 29 member banks in the HMBANA network,[70] which are regularly assessed to ensure adherence to the guidelines.[27]

Potential donors are screened via questionnaire and certified laboratory blood test, and may also be asked to provide written consent from a licensed health care professional. If approved, the donor's HM is logged into a database and stored in a freezer until processing, which involves thawing and decanting raw milk from individual storage containers, pooling milk from one or more donors, and then bottling pooled milk. Some milk banks also choose to measure macronutrients via human milk analyzer (HMA), but that step is optional. All DHM intended for use in the NICU undergoes Holder pasteurization (62.5°C for 30 minutes), which eliminates pathogens while still preserving many of the beneficial compounds in HM.[32,71] Pasteurized DHM is then screened for pathogens before being frozen and sent to the NICU.

Environmental Scan of HMBANA Banks

To learn about variations in DHM processing methods, we conducted an environmental scan of 9 banks. Visits to each milk bank were guided by an outline of questions and the observed processing methods were summarized in a matrix for comparison. While all 9 milk banks followed the HMBANA guidelines, there were individual variations in how some steps were done, as well as the terminology used to describe some steps in the process. We then developed a common language in order to internally describe observations from the field, then diagramed common DHM processing steps in a flow chart. Included in the Appendix are: (A) list of terms and definitions, (B) flow chart of DHM processing, (C) interview guide used during the environmental scan, and (D) matrix summarizing practices observed at each milk bank.

We identified the following processes as steps with the greatest potential to impact the distribution and retention of macronutrients: thawing, decanting, pooling, and mixing during bottling. The impact of the steps prior to thawing (e.g. storage and freezing) and after bottling (e.g. pasteurization) have been thoroughly investigated, and thus are not included. The sections below provide additional information about thawing, decanting, pooling, and mixing during bottling, including guidelines from HMBANA, observations from the environmental scan, an overview of relevant studies, and gaps in the literature.

The Impact of Thawing

HMBANA guidelines recommend gradual thawing in a refrigerator (although procedures for thawing in a water bath or non-refrigerated location are also provided) and that temperatures should be monitored to ensure HM does not exceed 7.2ºC.[27]

A study by Chan et al[72] investigated the impact of different thawing methods on fat content of HM. Each sample of fresh preterm HM (n = 17) was equally divided into three plastic tubes and stored at -20ºC for up to 3 weeks. Each tube contained 40–100 mL, and similar volumes were used in each thawing method, but it is unknown if each tube is a representative sample because the mixing method was not stated. One set of samples was thawed at room temperature (2.5–4.25 hr at 20ºC), another set was thawed in a water bath (12–30 min at 50ºC and swirled every 5 min), and the third set was divided between refrigerator (24–46 hr at 4ºC, n = 12) and microwave (1.5–2 min and swirled every 30–45 sec, n = 5). After no ice crystals remained, samples were heated in a water bath (5 min at 40ºC), vortexed, and measured by creamatocrit (CMCT). Compared to thawing in the refrigerator, average post-thaw CMCT was 18% lower for the water bath ($p = 0.01$) and 31% lower for the microwave ($p = 0.008$), but not significantly different for room temperature. The two major limitations of this study are the uncertainty of equal fat distribution in the plastic tubes due to lack of information about sample mixing, and the unbalanced comparison, so differences in fat may not be from thaw stage alone, but also due to differences in the HM.[72]

A study by Handa et al[73] compared characteristics of HM thawed using wet and dry methods. Fresh HM samples (n = 40, 100 mL each) were divided and stored at -20ºC for 7 days, but the storage container was not disclosed. HM was thawed in either a water bath or a dry bath 10 minutes at 37ºC, and subsequently allowed to sit at room temperature for 4 hours. Aliquots were removed at each step and stored at -80ºC until analysis of fat (gravimetric), protein (Bradford), IgA (ELISA). No significant differences were found when comparing the outcomes of wet vs dry, nor where there any significant differences when comparing the outcomes between processing steps. It should be noted that the temperature of HM during the last step (room temperature) ranged 20–30ºC, which exceeds limits in HMBANA guidelines, thus any conclusions drawn from this research may not be applicable to HM banking. Additionally, no information about mixing procedures were including in this article, thus it cannot be assumed that the aliquots were representative samples.[73] The importance of mixing is discussed in a later section.

A study by Vieiera et al[74] also compared the impact of two different thawing methods. Samples of raw HM (n = 57, 80 mL each) were stored in glass containers, pasteurized, equally divided into two samples, and frozen at -20ºC for 24 hours. Just like the previous studies, it is unknown if each tube is a representative sample because the mixing method was not stated. One sample was thawed in a water bath for 10 minutes at 37ºC (“slow thaw”) and the other was thawed in a microwave for 45 seconds (“quick thaw”). Macronutrients were measured via Milkoscan for raw HM, pasteurized HM, and thawed HM. Fat and protein contents were significantly different between steps, but not between thaw methods. Post-thaw, mean fat content was 7.8% lower than raw milk ($p < 0.001$) and mean protein content was 5.8% lower than raw milk ($p < 0.001$). Further statistical analysis identified pasteurization as the step where most nutrients were lost ($p < 0.001$), which is in opposition to the results of most studies.[32] Fat was the most variable macronutrient, but considerably lower than what is typically seen in the literature (2.2±1.5 g/dL at baseline). Conversely, mean protein content was similar to what is typically seen in the literature. Aliquoting technique was not described, and the authors make no relevant speculations regarding the abnormal macronutrient content.[74]

Thawing methods with different temperatures were assessed in a study by Thatrimontrichai et al.[75] Fresh HM samples (n = 90, 60 mL each) were divided in to three 20mL aliquots, with one used to measure baseline fat content (Gerber) and the other two stored in polypropylene (PP) plastic containers at -20ºC for 30 days. One set of aliquots was thawed in a refrigerator (4ºC for 24 hours) and the other was set was thawed in a water bath (37ºC for 30 min). The average baseline fat content (3.0±1.0 g/dL) is on the low end of what is seen in the literature. HM thawed via water bath lost an average of 0.1 g/dL more fat than HM thawed in a refrigerator ($p = 0.02$), with a mean decrease from baseline of 10.1% in the water bath ($p < 0.0001$) and 7.4% in the refrigerator ($p = 0.0001$). The authors hypothesize that the greater decrease in fat was likely attributed to the different temperatures, with the heat from the water bath allowing for liquid fat to cling to the container — and note this difference was visibly observed during the study.[75] There does not appear to be a consensus on the recommended method for thawing HM, which are summarized in Table 3.

Table 3. Summarized Results of Studies on Thawing Human Milk.

	Fridge (4ºC)	**Ambient (20ºC)**	**Water Bath**	**Micro-wave**	**Container Material**
Chan et al[72]	✓	✓	× (50ºC)	×	PE
Vieiera et al[74]	NA	NA	✓ (37ºC)	✓	Glass
Handa et al[73]	NA	NA	✓ (37ºC)	NA	Unknown
Thatrimontrichai et al[75]	✓	NA	× (37ºC)	NA	PP

Note: ✓ = recommend; × = do not recommend; NA = not assessed. For container material, PE — polyethylene; PP — polypropylene.

The two studies that assessed thawing in a refrigerator both recommend that method, but there was disagreement regarding the impact of thawing at 37ºC and above — although the implications derived from those studies may not be fully applicable to milk banks. In addition to the limitations described above, observations from the environmental scan indicate most DHM is thawed using various combinations of room temperature and refrigerator methods, staying within the constraints of the guidelines. Some milk banks did not allow the DHM to reach a completely liquid state prior to decanting, which can help reduce the amount of DHM lost from leaky bags — a common occurrence when bags are overfilled by the donor. Additionally, nutrients can also be affected by the material of the storage container, although much of the research has focused on containers used in the hospital setting and are not representative of what is seen in milk banks.[76,77]

The Impact of Decanting

Decanting is the process of pouring thawed HM from the original storage container into a larger pooling container; and is a function of two components: thaw stage and container type. Milk banks accept HM donations in two types of storage containers — plastic bags and hard plastic containers — the bags are typically polyethylene (PE, which is a more pliable plastic), and hard containers are typically polypropylene (PP, which is a more rigid plastic). Bags are more common, although the use of hard containers is gaining popularity because they can be sterilized and reused (and thus reduce waste), plus some HMBANA banks will provide them to donors upon request.[78,79] Unlike hard containers, bags can be manipulated (e.g. squeezing, folding) to help remove any remaining contents after the majority of HM has been poured out. One common objection to bag manipulation during decanting is a concern over contaminants from the outside of the bag entering the DHM. In our environmental scan, we observed that each milk bank has a pre-defined thaw state (ranging from mostly ice to completely liquid), as well as a rule for bag manipulation (always, never, allowed but not typically done). Visual inspections of decanted DHM bags during the environmental scan indicate that thaw stage and bag manipulation may impact the amount of DHM (and thus amount of nutrients) retained at this step. The topic of decanting has not been investigated, and no recommendations about decanting are included in the HMBANA guidelines.[27] Related studies in the literature may only decant partial volumes of HM via pipette (versus completely emptying the container), and few use storage bags (which are common in milk banks).

A study by Janjindamai et al[80] investigated the effect of two different HM storage containers on fat and bacteria content. Each sample of fresh HM (n = 90, 80 mL each) was mixed and equally divided into two hard PP containers and two flexible PE bags, frozen (-20ºC) for 30 days, then thawed in a water bath (37ºC for 30 min) and mixed prior to analysis. Pre– and post–thaw measurements were obtained for fat (Gerber method) and bacteria (plate cultures). No pathogenic bacteria were found, and fat loss was similar between the two container types, but fat loss from baseline was significant for both methods (approximately 0.3 g/dL, $p < 0.001$ for both). However, a systematic review from Gao et al[81] concluded that little fat is lost during processing (frozen storage, thawing, pasteurization), and asserts that studies reporting ≤ 10% loss are likely related to methodology (e.g. fat clinging to containers, unrepresentative samples from insufficient mixing) and are not true fat loss. Janjindamai et al state HM was “aseptically mixed” (which should indicate that each of the four aliquots have a similar fat content), the mean fat of fresh HM in the hard plastic was about 0.1 g/dL lower than fresh HM in the bag (p

= 0.009).[80] Also, more fat can cling to container walls when the ratio of container surface area to volume is large,[82] which likely occurred in this study, since each container had around 20 mL HM (minus what was removed for fat and bacteria testing).[80]

A study by Chang et al[83] examined the impact of container type on macronutrients, using nine unique containers (5 bags and 4 bottles). HM donations (n = 42) were combined to form pools (280 mL and 1–2 donors each), homogenized, analyzed for macronutrients with an HMA, and the remainder was equally divided amongst the containers (30 mL each). The samples were stored at -20ºC for 2 days, thawed at 4ºC for 12 hours, and homogenized again prior to macronutrient analysis via HMA. For all containers, fat was reduced by 8–9% ($p = 0.02$), CHO was increased by about 1% ($p = 0.001$), and protein was increased by 4–8% ($p = 0.021$), but the change in total energy was not significant ($p = 0.069$). The change in nutrient content between containers was not significantly different. Details about how the HM was removed from the container are not provided, so application of these results to the milk bank processing may be limited.

Overall, the best method for optimizing the removal of HM from a storage bag has yet to be elucidated. The issue of container material is also relevant in later steps of processing, such as pooling and mixing during bottling. During those steps, single samples of DHM are combined into a large pooling container and may be stored at refrigerated temperatures for several hours prior to bottling. HMBANA guidelines state that processed HM should be stored in food-grade glass or plastic that is capable of withstanding freezer and Holder pasteurization temperatures.[27]

Some studies about container material have storage conditions similar to what would be seen in a milk bank prior to bottling (short-term, in the refrigerator). For example, a 1981 study by Goldblum et al[84] compared the impact of three containers (glass, PE bags, PP containers) held in a refrigerator up to 24 hours on the retention of bioactive compounds The authors concluded that no container yielded superior retention, but recommended PP containers over glass containers and PE bags due to ease of handing (e.g. glass may break, bags may spill).[84] Williamson and Murti[85] conducted a study with similar storage conditions, except using glass and stainless steel containers. They concluded that cells may more readily adhere to the walls of steel containers due to microscopic differences in texture (steel was not as smooth as glass).[85] However, neither of the above studies reported the size/shape of the container or volume of HM, which is important because the potential for adhesion increases as the ratio of container surface area to HM volume increases.[82]

The Impact of Pooling

Pooling of DHM helps to mitigate potential sources of nutrient variation. Unlike the dairy industry, which uses enormous vats capable of holding several thousand gallons,[86] milk banks in the HMBANA network are limited to smaller pools of milk totaling a few thousand ounces per day, which in turn limits the number of donors that can be pooled together. Previous versions of the guidelines stated that up to 10 donors could be used to create a pool of DHM, but the current guidelines simply defines a pool as "more than one donor."[27] Despite this definition, a recent study of DHM from a single HMBANA bank found 41% of sampled pools contained only one donor.[45] This may be due to other factors when choosing donors for a pool, such as pump date, lactation stage, donors with dietary restrictions (e.g. dairy-free) or whose milk has previously tested positive for contamination. Some milk banks choose donors to combine based on macronutrient measurements via HMA (called "target pooling"), although macronutrient testing is not required per the guidelines.[27] The studies below focus on the number of donors combined in the final pool that is ready to be bottled.

An investigation by John et al[44] used a large dataset of HM composition from over 500 women to simulate 2,000 random pools of up to 5 donors per pool. The results indicated that increasing the number of donors in pool decreased fat and protein variability.[44] Halleux and Rigo[87] compared variability in single-donor (n = 138) and multiple-donor (n = 224) pools in Belgium, although the specific number of donors in the multiple-donor pools was not provided. Variability was calculated as a percent using mean absolute difference (defined as "the mean value of the absolute difference between all individual values and the mean")[87] and macronutrients were measured via HMA. Compared to multiple-donor pools, single-donor pools had significantly higher variability for total energy (6.9% vs 5.3%, $p < 0.05$) and protein (19.3% vs 13.5%, $p < 0.05$), but, unlike the previous study, fat variability (around 10%) did not differ by the number of donors in a pool.[87]

Non-HMBANA banks may have different pooling practices, as indicated by a study from Meredith-Dennis et al.[42] The study compared DHM from one HMBANA bank (n = 3; 3 donors per pool) with DHM from two non-HMBANA banks (n = 3 each; one bank used 200 donors per pool and the other used 250), and found the 3-donor pools had higher variability (as relative standard deviation) for fat (10.5 compared to 0.8 and 5.4, $p \leq 0.05$) and protein (26 compared to 4.8 and 19.2, $p \leq 0.05$), than the 200– and 250–donor pools, respectively.[42]

A multi-faceted study by Stoltz Sjostrom et al[30] compared the macronutrient contents of single-donor (n = 225) and multiple-donor (n = 129 samples, 2–4 donors per pool) pools in Sweden using an HMA. Although variability was not assessed between the groups, a comparison of mean macronutrient values yielded results in opposition to other studies. Compared to multiple-donor pools, macronutrients in single-donor pools were higher for total energy (69±9 vs 66±6 kcal/dL, $p = 0.001$), fat (3.6±0.9 vs 3.4±0.5 g/dL, $p = 0.001$), and protein (1.5±0.4 vs 1.3±0.2 g/dL, $p < 0.001$). However, these findings may not be applicable to DHM in the US, since donations of HM in Sweden are limited to no more than 3 months post-partum.[30]

Overall, more information is needed in order to determine if there is an optimal number of donors that should be used in a pool, especially when target pooling is not done.

The Impact of Mixing During Bottling

From dairy industry research, it is known that time and temperature impact the kinetics of fat separation.[88] Milk left undisturbed will separate into fat and skim layers,[89] which may result in an uneven distribution of nutrients, especially fat, during bottling.[90] HMBANA guidelines do not provide information on how to mix DHM,[27] and studies of mixing methods in the milk bank setting are lacking.

Research about mixing HM has been conducted in the clinical setting using ultrasonication to mix small volumes of HM. At the time of the environmental scan, no HMBANA banks were using ultrasonication to mix large pools of DHM. There is evidence supporting the clinical use of ultrasonication during feeding preparation of HM to improve fat delivery and absorption for preterm infants fed via gastric tube. For example, a balance study by Thomaz et al[91] compared the effect of ultrasonication on fat absorption in VLBW preterm infants (n = 10) fed pasteurized HM (MOM and DHM combined) via orogastric tube. HM was ultrasonicated in individual daily batches (up to 200 mL/kg/d) at a rate of 6 seconds/mL (other settings not stated) while held in an ice bath. Over 12 days (ultrasonication occurred on 6 randomly assigned days), average fat absorption was 8% higher when HM was ultrasonicated.[91] The reason for this improved fat digestion was elucidated in a similar study de Oliveira et al,[92] who measured additional outcomes. The pasteurized DHM (PDHM) in this study was subjected to 3 intervals of indirect ultrasonication (the PDHM sample was placed in a water bath and the water was ultrasonicated, as opposed to inserting the probe directly into the PDHM sample), each for 5 minutes with 30 seconds of rest (other settings not stated). When the preterm infants (n = 8) were fed ultrasonicated DHM, the rate of meal half-emptying was an average of 8 minutes longer ($p < 0.001$) and the degree of lipolysis was an average of 3.6% higher ($p < 0.01$), thus the authors also concluded that ultrasonication improved fat digestion.[92]

A limitation of some ultrasonication studies, including those listed above, is the lack of detail regarding the ultrasonicating device (which have a variety of attachments and settings). Absence of this information poses difficulty for replication and creation of an ultrasonication protocol. For example, a study by Czank et al[93] ultrasonicated HM at a rate of 5 seconds/mL (5 minutes total duration) and at 70% amplitude (60 W), but Garcia-Lara et al[94] used a rate of 1.5 seconds/mL and 75% amp for various volumes of HM. Martinez et al[95] used rates of 4–8 seconds/mL at intensities of 4–8 (0–10 scale), and de Oliveira et al[92] used a pulse setting to ultrasonicate 60–75 mL samples for 3 rounds of 5 minutes with 30 seconds in between.

It should be noted that ultrasonication can drastically increase the temperature of HM beyond limits stated in the HMBANA guidelines.[95] Christen et al[96] found a linear trend between time and temperature ($R^2 = 0.99$, $p < 0.05$), where 12 mL HM reached 65°C after almost 2 minutes of ultrasonication at 100% amplitude. Also, duration of exposure, not intensity, was observed to have greater impact on temperature, and the researchers recommended the use of cooling system when ultrasonicating.[96]

It is known that HM needs to be mixed to ensure a uniform distribution of nutrients. In the field, we observed manual and mechanical methods of mixing. Manual mixing used intervals of hand swirling and hand pouring — different combinations intervals were observed, ranging from swirling before each pour to swirling then pouring up to three bottles. Mechanical mixing used continuous stirring with a device (e.g. stand mixer, magnet, and/or oscillating plate) and occurred simultaneously during pouring (via dispensing pump). The initial degree of agitation varied but decreased as the volume of HM decreased. The potential differences between mixing methods on nutrient distribution in DHM remains to be elucidated.

Conclusion

The previous sections of this paper have demonstrated the relationships between HM macronutrients and growth outcomes in the preterm infant, and illustrated the negative impact of low energy and protein consumption. Many hospitals use feeding protocols that assume a standard macronutrient content in DHM, but evidence suggests the actual macronutrients are highly variable. This may be partly attributed to some processes in HM banking, and there exists a need for specific research to address the current gaps and weaknesses in the literature. Specifically, research is needed on how to optimize the retention and distribution of nutrients in DHM during the processes of thawing, decanting, pooling, and mixing during bottling.

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CHAPTER III

FAT AND PROTEIN VARIABILITY IN DONOR HUMAN MILK AND ASSOCIATIONS WITH MILK BANKING PROCESSES

Abstract

Background: Donor human milk (DHM) is the preferred source of nutrition for preterm infants when mother's own milk is not available. Evidence suggests that macronutrients in DHM are highly variable, and the impact of milk banking processes on macronutrient variability in DHM remains largely unknown. *Research aim*: To gain a better understanding of fat and protein variability in DHM and assess potential relationships with milk bank processing methods. *Methods*: Samples of raw, pooled DHM were obtained from 20 milk banks (n = 300), and the following processing variables were recorded for each sample: if macronutrient analysis was used to select donors for the pool (target pooling; yes/no), number of donors per pool, pooling container material (glass/plastic/other), and mixing during bottling method (manual/mechanical). Fat content was measured using creamatocrit and protein content was measured using BCA. Homoscedasticity was assessed and magnitude of the spread was quantified. *Results*: Fat content ranged 1.9 to 6.1 g/dL (n = 298) and protein content ranged 0.7 to 1.4 g/dL (n = 300). Variability in fat was significantly lower in samples that had been target pooled ($p = 0.04$), contained more donors per pool ($p < 0.001$), and had been mixed mechanically ($p < 0.001$). The variability in protein was significantly lower in samples that contained more donors per pool ($p = 0.001$). In a stratified analysis, increasing the

number of donors per pool only reduced nutrient variability in samples that were not target pooled. *Conclusion*: For milk banks that do not target pool, using a greater number of donors in a pool may reduce fat and protein variability. For milk banks that target pool, the number of donors per pool does not significantly influence macronutrient variability.

Introduction

Donor human milk (DHM) is the preferred source of nutrition for preterm infants when mother's own milk is not available.[1–3] The use of DHM in neonatal intensive care units (NICU) is increasing, with a 74% increase between 2011 and 2015.[4] NICUs receive DHM from milk banks, after the milk has undergone a variety of production processes to ensure a safe product. Research on DHM from milk banks in North America suggest that the macronutrient content is highly variable, although many studies are limited by a small number of samples,[5–7] or the samples were obtained from only one milk bank.[8–10] A systematic review of DHM found a collective range of 1.1 to 7.4 g/dL for fat, 5.5 to 8.6 g/dL for lactose, 0.8 to 2.2 g/dL for protein, and 43 to 86 kcal/dL for energy.[11] For preterm infants, intakes of energy and protein are positively associated with growth,[12–16] suggesting that efforts to obtain a more consistent profile of macronutrients in DHM are warranted.

The American Society for Parenteral and Enteral Nutrition[17] recommends that DHM be obtained from a milk bank following evidenced-based guidelines, such as those published by the Human Milk Banking Association of North America (HMBANA), a network of non-profit milk banks in the United States and Canada. The HMBANA guidelines provide standards for several areas of milk bank operations, including donor screening and milk processing.[18] At a high level, DHM processing entails thawing milk, combining milk from multiple donors into a pool, bottling, pasteurizing, and testing. Some milk banks choose to analyze a donor's milk for macronutrients and strategically combine donors to reach macronutrient targets (targeted pooling). It is known that macronutrients in human milk are variable and change over time.[19] Evidence suggests that target pooling may help mitigate the impact of individual macronutrient variations and produce DHM with less macronutrient variability, though current milk banking guidelines do not require targeted pooling.[20] Increasing the number of donors per pool is another strategy to reduce nutrient variability.[10] The guidelines do not limit the maximum number of donors in a pool or suggest a specific range that should be used.[18] Studies using pooled DHM have reported the number of donors ranged from 3 or 4 up to 10,[20–22] but two recent studies found pools with 1 to 5 donors.[9,10] To date, there have been no large-scale studies of DHM involving multiple milk banks. Further, there is limited research into how processes within milk banking may impact the distribution of nutrients in DHM.

The primary objective of this study was to describe the variability in protein and fat in a large set of DHM samples from multiple milk banks and assess potential relationships with two main processing factors — target pooling and the number of donors in a pool. These processing variables were chosen because current data in the literature suggest there may be a significant impact on nutrient variability.[9,10,12,23] The secondary objective was an exploratory analysis that included two additional processing variables — material of the pooling container and method of mixing during bottling —

and their impact on fat and protein variability.[24–29] Fat and protein were selected for preliminary analysis because of their role in supporting preterm infant growth in the NICU.[2,30] We hypothesized that variances in fat and protein would be significantly reduced in DHM when milk banking production processes used target pooling or increased the number of donor per pool.

Materials and Methods

This study was reviewed by the University of North Carolina Greensboro (UNCG) Institutional Review Board and categorized as non-human subject research (protocols 17-0140 and 17-0523). After approval from the HMBANA Board of Directors and the HMBANA Research Committee, all milk banks (n = 27) in the HMBANA network were contacted about the study via email. Milk banks that elected to participate in the study were assigned an ID code (one letter, A–T) and mailed a package containing sampling instructions, a data collection log, and 15 sterile sample cups (03008-7TN; Starplex Scientific Corp, Cleveland, TN USA). Digital versions of the instructions and data collection log were also provided.

Sample Collection

Sample collection cups were pre-labeled with a number (1–15) and the milk bank's assigned ID code. Instructions were to collect at least 2 oz from the first pour of 15 unpasteurized pools of DHM intended for a NICU. Once collection began, samples were to be obtained from each consecutive, unique pool of DHM — excluding chylothorax/skim and non-NICU pools (e.g. outpatient, well-baby) — and poured into the cups using the typical pouring method (e.g. dispensing pump, hand pour). Samples were to be stored in a freezer until all 15 samples were ready to be shipped on dry ice via overnight delivery.

Data Collection Log

The collection log was a 1-page document designed to quickly record the following processing variables of each sample: date the pool was prepared, if macronutrient analysis was used to select the donors for this pool (target pooling; yes/no), number of donors in the pool, material of the container that held the pool while it was being bottled (glass/plastic/other — specified), and method used to pour milk into bottles (hand/pump). The latter variable was used to assess the method of mixing during bottling (manual/mechanical). Based on observations in the field, including separate variables for pouring and mixing was redundant because pouring method was analogous with mixing method — milk banks that poured by hand also mixed by hand (via intervals of swirling the container) and milk banks that poured via dispensing pump also used a device to continuously mix the DHM (e.g. magnets, stand mixer, and/or oscillating plate). “Pouring method” was used on the collection log instead of “mixing method” because pouring is better associated with bottling, and DHM is mixed during other steps in processing. There was also a “notes” section for attributes about the pool that may be tracked by the milk bank (e.g. term or preterm milk, dairy-free, calorie/nutrient content, etc.). Instructions were to record an entry in the log (using the row corresponding to the sample ID on the cup) after each sample was collected, and return the completed log with the frozen samples to our lab at UNCG.

Sample Handling

Upon arrival to our lab at UNCG, samples were checked for possible temperature abuse and stored at -20°C until processing. Each set of samples (15 cups from one milk bank) was processed at a time and de-identified data were recorded according to the assigned ID code. One researcher completed all processing and analyses.

Thawing occurred in a Precision Shaking Water Bath 15 (SWB; TSSWB15; Thermo Fisher Scientific, Newington, NH USA) at 55 rpm and 35°C for at least 30 minutes, until no ice crystals remained and temperature of the DHM was within 20–24°C. A preliminary investigation determined that magnetic mixing on a stir plate (11-498-7SH; Fisher Scientific, Bohemia, NY USA) for 5 minutes at a moderate speed (4–5 on a scale of 1–10) was necessary to obtain representative aliquots from each cup. After 5 minutes, mixing continued while 1-mL aliquots were pipetted into 1.5 mL microtubes and stored at -20°C until analysis. Temperature was monitored at multiple points during processing using a digital thermometer (11779725; FisherBrand, Goteborg, Sweden). For all analyses, aliquots from the same milk bank were tested as a set after thawing for 10 minutes at 30°C using a Digital Heating Cooling Drybath (88880029; Thermo Fisher Scientific, Waltham, MA USA).

Sample Analysis

Fat was measured using the creamatocrit method by Lucas et al, which uses the percentage of cream to calculate the fat content of HM.[31] Meier et al. found creamatocrit to have high intra– and inter–user reliability (all mean differences < 1%), and ability to measure fat content similarly to other common laboratory procedures.[32] In a comparison of three methods for measuring fat, Du et al found creamatocrit to be precise (coefficient of variation, CV = 3.9%) and have a close correlation ($R^2 = 0.995$) with the gravimetric method (a gold standard reference method), despite under-reporting by 0.3–0.6 g/dL.[33] Although this technique is more operator-dependent than other tests,[34,35] a preliminary investigation illustrated that the researcher was able to consistently achieve low CVs (< 3%) by vortexing the microtubes (as opposed to manual inversions) and using a flatbed centrifuge to create better distinguished fat layers. Specifically, a microtube of DHM was vortexed for 10 seconds at level 5 intensity on a Vortex Genie 2 (12-812; Fisher Scientific, Bohemia, NY USA) prior to filling each capillary tube. The capillary tubes were spun for 10 minutes at 11.2 x 1000 rpm on a Zip-IQ PCV Centrifuge (ZiC-24HD-75T3; LW Scientific, Lawrenceville, GA USA), as determined by Miller et al,[34] then measured using a Creamatocrit Plus (100-146; EKF Diagnostics, Boerne, TX USA). Creamatocrit values were converted to fat (g/dL) with the equation determined by Meier et al[32]: $(3.968 + 5.917 \times \text{creamatocrit}) \div 10$.

Protein was measured via Pierce bicinchoninic acid assay (BCA; 23225; ThermoFisher Scientific, Rockford, IL USA), which has been validated for HM,[36] although it likely overestimates by as much as 30%.[34] This technique is based on the reduction of Cu^{+2} to Cu^{+1} via biuret reaction, where Cu^{+1} is colorimetrically detected when it reacts with a BCA reagent. A set of samples was thawed, diluted 1:10, and loaded in a 96-well plate with standards made using bovine serum albumin. After adding the BCA working reagent, the plate was incubated for 30 minutes at 35°C, cooled on ice for 10 minutes, then read using a spectrophotometer (7091000; BioTek, Winooski, VT

USA) — the purple-colored product has a strong absorbance at 562 nm, the intensity of which is proportional to protein concentrations ranging from 20 to 2000 µg/mL. Protein (g/dL) was quantified with a standard curve and adjusted using the formula established by Keller and Neville for comparison to Kjeldahl methods.[36]

Statistical Analysis

The independent variables included: milk bank ID (letters A–T); two primary processing variables, target pooling (yes/no) and number of donors per pool (number); and two secondary processing variables, container material (plastic/non-plastic) and mixing during bottling (manual/mechanical). The dependent variables were fat (g/dL) and protein (g/dL).

Statistical analysis was conducted using R software (version 3.5.2; R Foundation for Statistical Computing, Vienna, Austria). Case influence statistics (studentized residuals and Cook's distance) were used to determine outliers. Descriptive statistics (n, mean, median, standard deviation, minimum, maximum, and quartiles) for fat and protein were calculated for the full dataset and for each processing variable. A chi-square analysis was performed for all pairwise combinations of processing variables to compare distributions. Differences by processing variables were assessed with ANOVA. Since the primary objective of this study was to assess whether target pooling and number of donors per pool influence the nutrient variability in DHM, we performed statistical tests to assess for unequal variances. When chi-square distributions were significantly different, stratified analyses were performed to control for differences in processing attributes. Homoscedasticity was assessed using the Fligner-Killeen test, and magnitude of the spread was quantified by sample variance (s^2). This test was also used for an exploratory analysis, which investigated the impacts of the secondary processing variables (container material and mixing during bottling) on the variance of fat and protein distributions. Linear mixed models were used to investigate the combined impact of all four processing variables on mean fat and protein content, with milk bank ID as the random intercept to control for clustering. Contributions to the overall model, including the fixed effects of the processing variables and the random effect of milk bank, were quantified.

Results

Twenty milk banks participated, each sending 15 samples for a total of 300 unique samples. Sample characteristics are given as a percentage of all 300 samples. Target pooling was used for 45% (n = 135/300) of samples, and the other 55% (n = 165/300) were not target pooled. One observation did not report the number of donors in the pool (and was omitted from analyses involving that variable), the other observations are as follows: 17% (n = 51/299) had 1-donor, 28% (n = 83/299) had 2-donors, 40% (n = 121/299) had 3-donors, 11% (n = 33/299) had 4-donors, and 4% (n = 11/299) had 5-donors. For 25% (n = 75/300) of the samples, a plastic container was used to hold the pool of DHM, and a non-plastic container was used to hold the other 75% (n = 225/300). Manual mixing was used for 59% (n = 176/300), while 41% (n = 124/300) were mixed using a mechanical device.

Fat was measured in duplicate and protein was measured in triplicate (average CV for replicate measures was 2.2% and 3.0%, respectively). Figure 1 maps the fat and protein content for all 300 samples, with two observations for fat (9.0 g/dL, 14.6 g/dL) identified statistically as influential outliers. Those samples were retested and yielded the same values for fat content, and thus omitted from additional analyses involving fat. All protein values were within physiological ranges, and no observations were omitted.

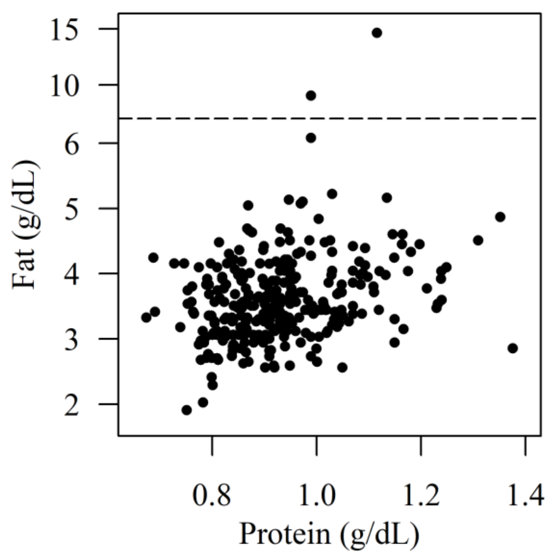

Figure 1. Fat and Protein Content of Donor Human Milk. Includes all samples (n = 300). Statistical outliers are plotted above the dashed line.

Descriptive Statistics

Descriptive statistics for the dataset (excluding the NA for donors per pool and two outliers for fat) are presented in Table 4. Fat content ranged 1.9 to 6.1 g/dL (n = 298), and protein content ranged 0.7 to 1.4 g/dL (n = 300). The chi-square distributions were significantly different for all pairwise comparisons of the processing variables ($p < 0.001$ for all). There was a significant difference in fat (mean ± standard deviation) by pooling container material (3.7±0.6 g/dL for plastic and 3.5±0.6 g/dL for non-plastic; $p = 0.007$). No other differences in mean fat or protein values were observed.

Table 4. Descriptive Statistics for Fat and Protein (g/dL) by Processing Variables.

Processing Variables	**Fat (g/dL) (n = 298)**	**Protein (g/dL) (n = 300)**
All Samples	3.6±0.6 (1.9–6.1)	0.9±0.1 (0.7–1.4)
By Target Pooling		
Yes (n = 135)	3.5±0.5 (2.6–5.2)	0.9±0.1 (0.8–1.3)
No (n = 165)	3.6±0.7 (1.9–6.1)	0.9±0.1 (0.7–1.4)
By Donors Per Pool		
1-Donor (n = 51)	3.5±0.7 (1.9–4.9)	1.0±0.2 (0.7–1.4)
2-Donors (n = 83)	3.7±0.6 (2.4–5.2)	0.9±0.1 (0.7–1.2)
3-Donors (n = 121)	3.6±0.6 (2.6–6.1)	0.9±0.1 (0.7–1.3)
4-Donors (n = 33)	3.5±0.5 (2.3–5.1)	0.9±0.1 (0.8–1.1)
5-Donors (n = 11)	3.4±0.3 (3.0–4.0)	0.9±0.1 (0.8–1.0)
By Pooling Container		
Plastic (n = 75)	3.7±0.6 (2.6–6.1)*	0.9±0.1 (0.8–1.2)
Non-Plastic (n = 225)	3.5±0.6 (1.9–5.1)	0.9±0.1 (0.7–1.4)
By Mixing Method		
Manual (n = 176)	3.6±0.7 (1.9–6.1)	0.9±0.1 (0.7–1.4)
Mechanical (n = 124)	3.5±0.5 (2.6–5.2)	0.9±0.1 (0.8–1.2)

Note: Data represent mean ± standard deviation (minimum – maximum). Differences between groups were evaluated with ANOVA. *$p < 0.05$ within a processing variable.

Impact of Target Pooling and Donors Per Pool on Fat and Protein Variance

The primary analysis investigated the impacts of target pooling and number of donors per pool on the variability of fat and protein content. For target pooling (Figure 2), variance was significantly different for fat ($s^2 = 0.43$ for No and 0.26 for Yes, $p = 0.04$), but not protein ($p = 0.78$). Regarding the number of donors per pool (Figure 3), variance was significantly different for fat ($s^2 = 0.55$ for 1-donor, 0.34 for 2-donors, 0.32 for 3-

donors, 0.29 for 4-donors, and 0.09 for 5-donors; $p < 0.001$) and for protein ($s^2 = 0.026$ for 1-donor, 0.016 for 2-donors, 0.011 for 3-donors, 0.008 for 4-donors, and 0.005 for 5-donors; $p = 0.001$).

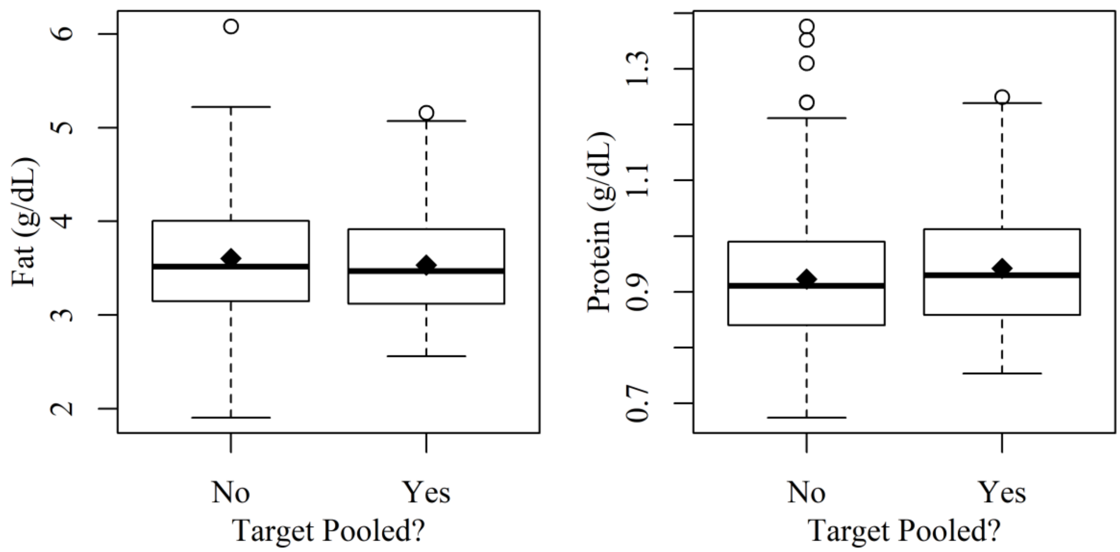

Figure 2. Distribution of Fat and Protein by Use of Target Pooling. Diamonds (♦) represents mean values; rectangles represent Quartile 1 to Quartile 3; solid lines represent median. Fligner-Killeen test of homogeneity of variances, p = 0.04 and 0.78 for fat (n = 298) and protein (n = 300), respectively.

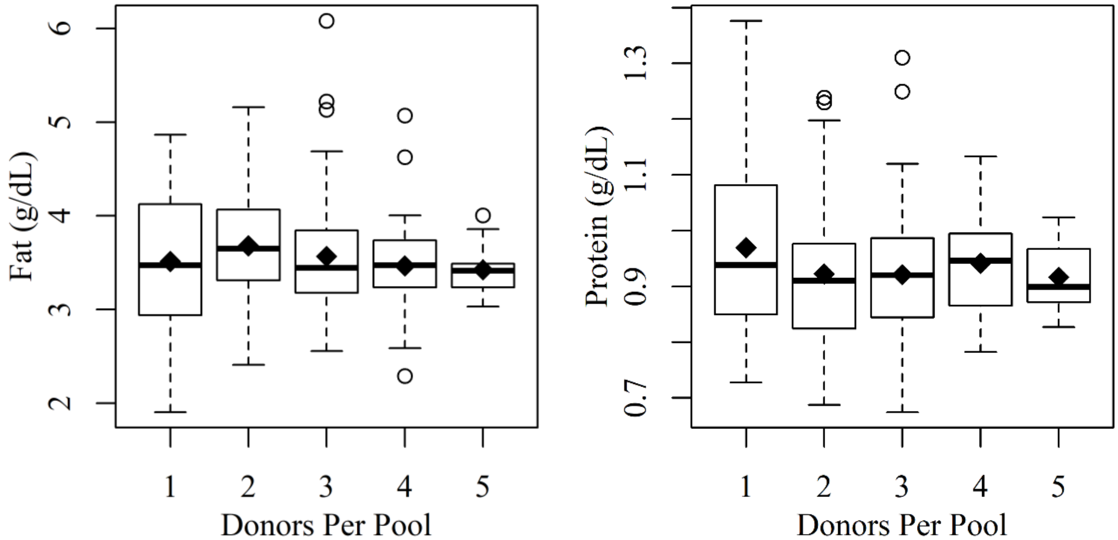

Figure 3. Distribution of Fat and Protein by Number of Donors in a Pool. Diamonds (♦) represents mean values; rectangles represent Quartile 1 to Quartile 3; solid lines represent median. Fligner-Killeen test of homogeneity of variances, $p < 0.001$ and $p = 0.001$ for fat ($n = 297$) and protein ($n = 299$), respectively.

Since there were significant differences in the chi-square distribution for number of donors per pool by target pooling (percent of samples not from a targeted pool: 59% for 1-donor, 53% for 2-donors, 45% for 3-donors, 73% for 4-donors, and 100% for 5-donors; $p = 0.002$), we performed a stratified analysis by target pooling. When target pooling was not performed (Figure 4), variance between the number of donors per pool was significant for both fat ($n = 163$; $s^2 = 0.65$ for 1-donor, 0.41 for 2-donors, 0.47 for 3-donors, 0.25 for 4-donors, and 0.09 for 5-donors; $p = 0.01$) and protein ($n = 164$; $s^2 =$ 0.035 for 1-donor, 0.011 for 2-donors, 0.012 for 3-donors, 0.007 for 4-donors, and 0.005 for 5-donors; $p < 0.001$). When target pooling was performed, variance between the number of donors per pool was not significant for fat ($n = 134$; $p = 0.27$) nor for protein ($n = 135$; $p = 0.30$).

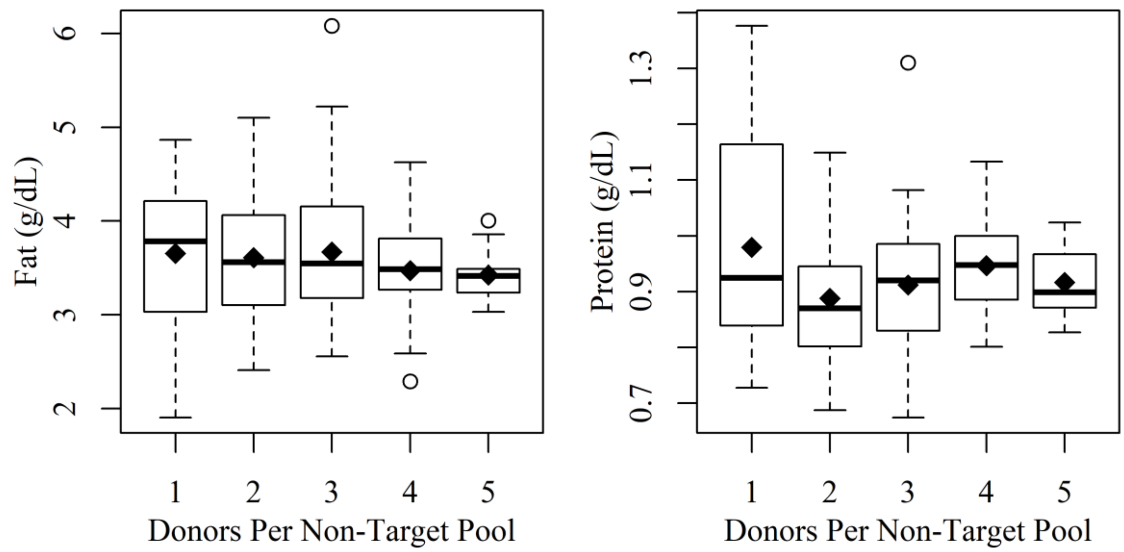

Figure 4. Distribution of Fat and Protein by Number of Donors in Non-Target Pools. Diamonds (♦) represents mean values; rectangles represent Quartile 1 to Quartile 3; solid lines represent median. Fligner-Killeen test of homogeneity of variances, $p = 0.01$ and $p < 0.001$ for fat ($n = 163$) and protein ($n = 164$), respectively.

Impact of Container Material and Mixing During Bottling on Fat and Protein Variance

The exploratory analysis investigated the impacts of the pooling container material and method of mixing during bottling on the variability of fat and protein content. For the material of the container, variance was not significantly different for fat ($p = 0.43$) or protein ($p = 0.36$). There were significant differences in the chi-square distribution for container material by target pooling (percent of samples not from a targeted pool: 40% of plastic and 60% of non-plastic; $p = 0.004$). When target pooling was not performed (Figure 5), variance between container materials was significant for protein ($n = 165$; $s^2 = 0.005$ for plastic and 0.017 for non-plastic; $p = 0.03$) but not for fat ($n = 164$; $p = 0.42$). When target pooling was performed, variances were not significantly different based on pooling container for fat (n = 134; p = 0.85) or protein (n = 135; p = 0.63).

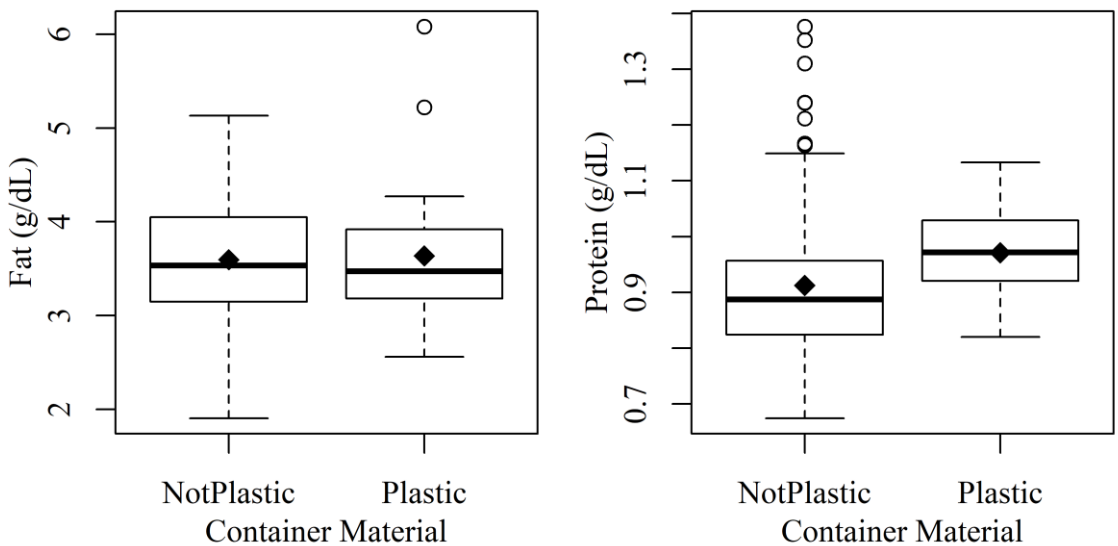

Figure 5. Distribution of Fat and Protein by Container Material in Non-Target Pools. NotPlastic — pooling containers made of glass or stainless steel; Plastic — pooling container made of plastic. Diamonds (♦) represents mean values; rectangles represent Quartile 1 to Quartile 3; solid lines represent median. Fligner-Killeen test of homogeneity of variances, p = 0.42 and p = 0.03 for fat (n = 164) and protein (n = 165), respectively.

Regarding the mixing method, variance was significantly different for fat (s^2 = 0.45 for manual and 0.22 for mechanical, $p < 0.001$), but not for protein (p = 0.44). There were significant differences in the chi-square distribution for method of mixing during bottling by target pooling (percent of samples not from a targeted pool: 68% of manual and 36% of mechanical; $p < 0.001$). When target pooling was not performed (Figure 6), variance between mixing methods was significant for both fat (n = 164; s^2 = 0.50 for manual and 0.17 for mechanical; $p < 0.001$) and protein (n = 165; s^2 = 0.018 for manual and 0.007 for mechanical; p = 0.008). When target pooling was performed, variances were not significantly different based on mixing method for fat (n = 134; p = 0.34) or protein (n = 135; p = 0.06).

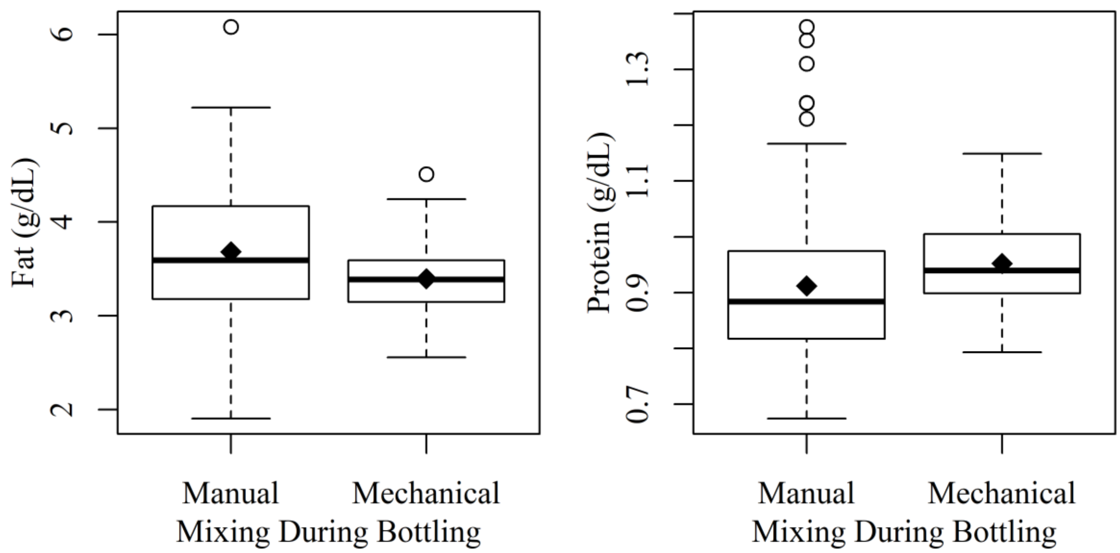

Figure 6. Distribution of Fat and Protein by Mixing Method in Non-Target Pools. Manual — pooling container is intermittently swirled by hand to mix the milk; Mechanical — pooling container is continuously mixed using a device (e.g. stir plate). Diamonds (♦) represents mean values; rectangles represent Quartile 1 to Quartile 3; solid lines represent median. Fligner-Killeen test of homogeneity of variances, $p < 0.001$ and $p = 0.008$ for fat (n = 164) and protein (n = 165), respectively.

Predicting Fat and Protein Content Using Linear Mixed Modeling

Linear mixed models were used to investigate if the combined impact of all four processing variables could predict fat and protein content. For both the fat and protein models, none of the predictor variables were significant except for container material in the fat model. The model for predicting fat had a total explanatory power of 9.0%, in which the fixed effects explained 4.6% of the variance and the random effect of milk bank explained 4.5% of the variance. The fat model's intercept was at 3.98 (SE = 0.15, 95% CI [3.67, 4.29]), and the effect of container material was significant ($B = -0.28 \pm 0.10$, 95% CI [-0.49, -0.06], $p = 0.02$). The model for predicting protein had a total explanatory power of 11.7%, in which the fixed effects explained 3.1% of the variance and the random effect of milk bank explained 8.6% of the variance. The protein model's intercept was at 0.97 (SE = 0.05, 95% CI [0.88, 1.06]).

Discussion

In independent analyses of milk bank processing factors, we observed that fat variability was significantly lower when: a macronutrient analyzer was used to create targeted pools; there was a greater number of donors per pool; and, DHM was mechanically mixed during bottling. We observed that protein variability was significantly lower when there was a higher number of donors in a pool. However, there were significant differences in the distribution of processing variables (as illustrated by bivariate chi-square distributions with $p \leq 0.004$), and stratified analyses revealed more nuanced findings. Specifically, the variance of fat and protein in target pooled samples was not significantly influenced by the number of donors per pool, mixing method, or pooling container. In samples that were not target pooled, the variance of fat and protein was reduced in pools with a greater number of donors and in pools that were mechanically mixed; the variance of protein was reduced in pools using a plastic pooling container.

Impact of Target Pooling

Our results indicate that target pooling via pre-pooling macronutrient analysis was associated with a decrease in fat and protein variability in DHM compared to non-target pooled samples. Milk banks that choose to perform macronutrient analysis will typically use an infrared (IR) analyzer. This device exposes a set volume of HM (ranging 1.5–45 mL) to IR radiation, and wavebands indicate chemical structure vibrations that are unique to each macromolecule.[37] Macronutrient analysis is not required by HMBANA, but 45% of our samples came from targeted pools, suggesting that analyzer use was relatively common. Recent research has shown that a variety of these devices are reliable and accurate for measuring fat and protein when used in a milk bank setting.[38]

IR was used to measure macronutrients in an observational study by Fu et al,[8] which found a wide range of macronutrients in target pooled DHM samples from one milk bank. Values ranged 1.5 to 4.5 g/dL for fat, and 0.3 to 1.4 g/dL for protein, although we observed higher minimum values in target pooled milk (2.6 g/dL for fat and 0.8 g/dL for protein). To obtain their samples, NICU technicians were instructed to save samples of "remaining milk"[8] that was left over after DHM had been removed from the bottle and used to create feedings. If only a small volume of leftover milk remained, the high ratio of surface area may provide more opportunities for fat to cling to the bottle, which translates to artificially lower fat (and thus energy) content. Additionally, the aliquoting technique was not consistent for each bottle of DHM (some were poured, others were pipetted), thus it cannot be assumed that the sample was an accurate representation of the DHM.[8]

Impact of Donors Per Pool

When samples were not target pooled, we found that increasing the number of donors per pool from 1 to 5 was associated with a decrease in variability for both fat and protein. This was expected per the Central Limit Theorem,[39] which states that an increased number of observations results in a decreased standard deviation, thus the pooling of multiple donors would help to mitigate potential sources of nutrient variation. Interestingly, the Central Limit Theorem did not apply when increasing the number of donors in targeted pools that used an analyzer to non-randomly select donors based on the macronutrient composition of their milk. Therefore, knowing the macronutrient composition of a donor's milk does not necessitate adding additional donors to the pool to drive down variability.

HMBANA defines a pool as "more than one donor,"[18] yet 17% of samples in this study were from single-donor pools. Similarly, a recent study by Young et al[9] used DHM from a milk bank in the HMBANA network, and 41% of the samples were from single-donor pools. To put the impact of donors per pool in perspective, a pilot study by Meredith-Dennis et al[7] reported the relative standard deviation in samples pooled with 3-donors ($n = 3$), 200-donors ($n = 3$) and 250-donors ($n = 3$). The authors found that the 3-donor pools had higher relative standard deviations for fat (10.5 compared to 0.8 and 5.4, $p \leq 0.05$) and protein (26 compared to 4.8 and 19.2, $p \leq 0.05$), than the 200– and 250–donor pools, respectively. This larger variance was even more pronounced for some bioactive compounds, such as immunoglobulin A (47.6 compared to 4.1 and 15.1, $p \leq 0.05$) and lysozyme (48.3 compared to 24.3 and 6.7, $p \leq 0.05$).[7] Despite the more extreme variability in the 3-donor pools, a study with a larger sample size is needed to better determine the effect of 200+ donors per pool. Regardless, it is unlikely that using such a high number of donors per pool is currently feasible for most HMBANA banks.

Our findings align with a study by John et al,[10] which simulated random pooling of up to 5 donors using a large dataset of HM composition from over 500 lactating women and compared to historical data on targeted pooling. The historical data was from pools created by a HMBANA bank, some of which were pools of only one donor (although the exact number of single-donor pools was not provided). The authors also concluded that increasing the number of donors in pool decreased variability.[10] These results are slightly different than a study from de Halleux and Rigo,[40] which compared variability (calculated as a percent using mean absolute difference) in single-donor and multiple-donor pools. Compared to multiple-donor pools, single-donor pools had significantly higher variability for protein ($p < 0.05$) but not fat. However, the specific number of donors in multiple-donor pools was not provided, and it is unknown if macronutrient analysis was used to create targeted pools. Similarly, Young et al[9] found that as the number of donors per pool increased, variability of protein decreased ($p = 0.014$), but variability of fat did not. However, there were only 11 pools with 3– to 4– donors, so the analysis may not have been adequately powered to detect differences in larger donor pools. Also, it was noted that donors were selected for pools based on the expiration date of the HM, but no mention of donor selection based on macronutrients.[9] Our novel finding suggests that nutrient variability in donor human milk can be reduced without necessarily adding more donors to the pool, if target pooling is used.

Impact of Container Material

Since the primary objective of this study assessed the variability of macronutrients, we did not expect to see significant differences in the means of macronutrient contents. However, the mean fat content of non-plastic containers was significantly lower than the mean fat content of plastic containers. This is likely due to one striking covariate — the number of donors in a pool. Specifically, 96% of single-donor pools (n = 49/51) and 100% of 5-donor pools (n = 11/11) were in non-plastic containers. Although not statistically significant, the mean fat content of single-donor and 5-donor pools was lower than the mean fat content of the entire dataset, which may help explain the significantly lower mean fat content for non-plastic containers.

When analyzing the entire dataset, we did not observe significant differences in fat or protein variability associated with the material of the pooling container. The lack of significant findings in the dataset as a whole aligns with the findings of previous studies — although previous research has not been within the lens of milk banking, instead investigating macronutrient loss (not variability) and in small containers that hold single samples of HM (not large containers for pools of HM). For example, a study by Chang et al[24] investigated the effect of nine unique HM containers (5 plastic bags, 3 plastic bottles, and 1 glass bottle) on macronutrient loss in 30 mL HM after frozen storage (-20ºC for 2 days), and found that the change in nutrient content between containers was not significantly different. A study by Goldblum et al[25] compared the impact of glass and plastic containers on the loss of bioactive compounds in single samples of HM held under storage conditions similar to what would be seen in a milk bank prior to bottling (up to 24 hours at 4ºC). The authors concluded that no container was superior regarding the loss of bioactive compounds, yet recommended plastic containers over glass containers due to ease of handing (e.g. glass may break).[25]

Our results, however, may be skewed because 75% of DHM samples (225/300) were from non-plastic containers, which included both glass (150/300) and stainless steel (75/300). A study by Williamson and Murti[27] compared the retention of biological components in HM stored in glass and stainless steel containers for up to 3 days at 4ºC. The authors concluded that at the microscopic level, steel containers were not as smooth as glass, and some biological components in HM (e.g. immunoglobulins and lysozyme) may more readily adhere to the walls of steel containers.[27] The increased popularity of steel containers in milk banking, combined with results from Williamson and Murti,[27] may warrant further investigation concerning the use of steel containers in the milk bank setting.

Impact of Mixing During Bottling

We observed a significant difference in fat variability associated with the method of mixing during bottling, but this was only observed in protein variability when the samples were from a non-targeted pool. The occurrence of two large outliers beyond the physiological levels for fat underscores the importance of adequate mixing, both immediately before and during bottling. From the dairy industry's research on gravity separation, it is known that time and temperature impact the kinetics of fat separation.[29] Fat in HM separates and forms a top layer, and fat globules can easily cling to the walls of the container — in order to ensure an aliquot with a representative fat content, the sample must first be properly mixed.[28] Studies investigating the mixing of HM have been done in a clinical setting with small milk volumes (e.g. one bottle < 120 mL), not in milk banks where pools typically range in volumes of 6 to 20 L.[41,42]

Linear Mixed Modeling

While statistical tests of unequal variance found that several milk bank processing factors were associated with reduced nutrient variability in DHM, a linear mixed model did not identify the four processing variables as significant factors that predict fat and/or protein content of raw, bottled DHM. Milk banks are a combination of multiple processing variables, only 4 of which were represented in this study. Those 4 variables created 40 unique combinations — more when the responses are not binary — and the results of the chi square distributions illustrated the wide variation in processing practices across the milk banks included in this study. The low power and lack of significance in the models may be partially attributed to the sample size of 300, which was not large enough to have a sufficient number of observations in all sub-group combinations.

Strengths and Limitations

A major strength of this study was the high participation rate (75% of banks in the HMBANA network), compared to previous studies with only 1 to 3 milk banks.[5–10] Additionally, we collected information on multiple processing factors which have not been systematically included in previous studies. Another strength was that measurements for fat and protein content were done by one researcher, with good CVs, and using established methods. The researcher also conducted a preliminary investigation to determine the sufficient method, duration, and speed of mixing necessary to obtain a representative aliquot from a sample of HM, which increases the reliability of study findings.

However, this was an observational study designed to establish relationships, not determine causation. There were significant differences in the distribution of the processing factors, thus it was difficult to attribute results to a single factor. We attempted to account for this with stratified analyses, which then led to more nuanced conclusions (e.g. variance was similar in target pooled samples, regardless of donor number, container, or mixing method; while variance in non-target pooled samples was significantly reduced by multiple processing factors), suggesting the need for more controlled research. The use of pouring method to assess mixing during bottling may also be considered a limitation of this study, but the instructions included clear descriptions of all variables, and there did not seem to be any confusion from the milk banks about how to document this variable. Although one milk bank did not pour by hand or by pump, but instead poured from a spigot on the container — these observations were recorded as "mechanical" because the DHM was continually mixed during bottling via magnet and stir plate.

Conclusion

In this large, multi-site study of 300 samples of DHM collected systematically from 20 milk banks, we observed 2-fold and 3-fold differences in the protein and fat composition of DHM. For milk banks that do not use macronutrient analyzers to target pool, fat and protein variability may be reduced by creating pools with a greater number of individual donors. For milk banks that use macronutrient analyzers to target pool donors, fat and protein variability was not influenced by the number of donors per pool, suggesting that the use of macronutrient analyzers to create targeted pools is a useful tool for controlling macronutrients in bottled DHM.

The findings of our study were based on fat and protein, and more research is needed to elucidate the impact of processing factors on the micronutrients and bioactive compounds in DHM. Our exploratory finding that mechanical mixing was independently associated with reduced variability in fat suggests more research on the impact of mixing using an experimental design is warranted. Additionally, other milk bank processing factors, such as the degree of thawing and technique for removing DHM from the original single sample container, should be included in future study designs.

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CHAPTER IV

METHOD FOR REMOVING THAWED HUMAN MILK FROM A PLASTIC STORAGE BAG IMPACTS FAT RETENTION

Abstract

Background: Milk banks have different methods for thawing and decanting human milk (HM). The impact of these processes on nutrient retention and bacterial contamination is largely unknown. *Research aim*: To determine how thaw stage and bag manipulation influence the retention of fat and number of aerobic bacteria colony forming units when decanting thawed HM from plastic storage bags. *Methods*: Lactating women ($n = 40$) in the Greensboro, NC area were recruited to provide fresh HM samples. Samples were divided into equal parts, placed in storage bags, and frozen for 2 months. Two thaw stages (ice/liquid) and the use of bag manipulation (yes/no) were assessed. Fat content was measured using ether extraction and total aerobic bacteria was measured using plate enumeration. Paired t-tests were used to compare the effects of thaw stage and bag manipulation on post-thaw fat content. Repeated measures ANOVA was used to compare the effect of bag manipulation on pre– and post–thaw bacteria. *Results*: Fat retention was not significantly different when thawing to a liquid versus an ice stage (mean difference = 0.10 g/dL; $n = 17$; $p = 0.07$). Decanting with bag manipulation retained more fat than decanting without bag manipulation, but only when HM was thawed to a liquid state (mean difference = 0.13 g/dL; $n = 11$; $p = 0.005$) and not when HM was thawed to an ice state ($p = 0.47$). Bag manipulation did not increase total aerobic

bacteria for either thaw stage ($p = 0.49$). *Conclusion*: For human milk frozen in a plastic storage bag, fat retention may be improved without increasing bacterial contamination by using bag manipulation during decanting when thawing to a liquid state.

Introduction

Donor human milk (DHM) is the recommended source of nutrition for preterm infants when mother's own milk is not available.[1–4] A recent Cochrane review found that while preterm infants fed formula had better growth outcomes than preterm infants fed DHM, they were also almost twice as likely to develop necrotizing enterocolitis,[5] a life-threatening disease of the gastrointestinal tract with an estimated mortality rate between 20 and 30%.[6] Despite findings of slower growth, many experts recommend DHM over formula due to the lower risk of necrotizing enterocolitis.[7–9] However, evidence also suggests that DHM is profoundly variable and may not align with the energy and macronutrients used for clinical reference methods in many standard fortification protocols.[10] Low intakes of energy and protein can negatively impact preterm infant growth,[11–15] and the loss of fat — a major component of total energy — during the production of DHM has not been fully elucidated.

Fat in human milk (HM) is packaged into milk fat globules (MFG), which vary in size and composition throughout the stages of lactation.[16] Over 98% of HM fat is in the form of triglycerides (TG), located in the core of the MFG.[17] A membrane surrounding the MFG (MFGM) prevents fat from coalescing and also protects the core from lipase enzymes, which remain active during refrigerated and frozen storage (4ºC and -20ºC, respectively).[18] The MFGM can be destroyed by freezing and thawing, making TG more susceptible to lipolysis, thereby increasing the amount of free fatty acids (FFA).[19] In fresh HM, FFA account for about 0.1% of total fat, but can increase to 3.6% within 2 to 5 months of frozen storage, and as high as 16.7% after two freeze/thaw cycles.[20] Increased levels of FFA have been associated with decreased pH, which may alter enzymatic activity[21]; as well as disagreeable flavors, which may lead to feeding refusal by the infant.[22] High FFA consumption has also been implicated in the pathogenesis of necrotizing enterocolitis.[23,24] Pasteurization and deep freezing (≤ -70ºC) have been effective inhibitors of lipase activity, thus preventing significant increases in FFA content.[25,26] Disruption of the MFGM by freezing and thawing also changes physical properties of HM. For example, FFA are able to compact more tightly than TG, which may falsely indicate fat loss with some testing methods (e.g. creamatocrit).[27] Similarly, MFGM destruction creates a less-stable emulsion where fat can more readily adhere to container walls, particularly small containers (e.g. microtubes).[19,28] Actual decreases in fat content during frozen storage are negligible,[21,26] but fat loss due to container adhesion is estimated to be 5–10%.[28] Some studies report fat loss can be mitigated by warming HM to around 37ºC,[18,28,29] while others report greater fat loss when thawing at 37ºC compared to thawing at 4ºC (refrigerator) or 20ºC (room temperature).[30,31]

Warming milk to 37ºC is not possible during the production of DHM. Guidelines published by the Human Milk Banking Association of North America (HMBANA) state that frozen HM can be thawed in a refrigerator, water bath or non-refrigerated environment, as long as the temperature of HM remains under 7.2ºC.[32] Studies investigating the impact of different thawing methods on nutrient retention either have conflicting results, exceed HMBANA temperature guidelines, and/or do not use storage bags or similar individual containers that are typically seen in milk banks.[30,31,33,34] There are currently no guidelines regarding how HM should be decanted from individual storage containers — including at what point in thaw cycle, and whether storage bags should be squeezed and manipulated to improve milk removal. It is possible that the process of decanting HM from storage bags may influence fat retention, and some bag manipulation techniques may introduce additional sources of bacterial contamination when the bag is squeezed and/or folded in attempt to remove as much HM as possible.

The purpose of this study was to assess how the method for decanting thawed HM from plastic storage bags influences the retention of fat and bacteria. Fat was selected because of its contribution to total energy, and thus its important role in supporting preterm infant growth in the NICU[16,35]; and bacteria was chosen to assess whether additional manipulation of storage bags increases bacterial exposure. We hypothesized that degree of thawing, and manipulation of storage bags will influence fat recovery, but will not influence total aerobic bacteria when decanting thawed HM.

Materials and Methods

Fresh human milk (HM) was collected for this study in order to best represent a milk banking scenario and capture the physical changes in HM that occur during storage. This study was approved by the University of North Carolina Greensboro (UNCG) Institutional Review Board (18-0303).

Participant Recruitment

From August 2018 through May 2019, lactating women in the Greensboro, NC area were recruited through social media and local breastfeeding groups. Women were eligible if they birthed a healthy, term (> 37 weeks gestation) infant who was 4–11 months of age at the time of enrollment. Additional criteria included: willing to record all food and beverage intake for 24-hours prior to their appointment; as well as ability to come to UNCG and donate approximately 90 mL of fresh HM using a new manual breast pump (44677-0505-20; Lansinoh, Alexandria, VA USA) that was provided to all study participants. Compensation included the breast pump as well as a $40 gift card.

Sample Collection

After scheduling an appointment, participants were sent an email that contained the following: a link to an instructional video about the breast pump, instructions for recording food and beverage intake during the 24-hours prior to the appointment, and a copy of the informed consent document. Participants were also asked to refrain from pumping or feeding from one breast at least 2 hours prior to their appointment.

Upon arrival to the scheduled appointment, the participant was greeted by a researcher, taken to a private room in the Cemala Foundation Human Nutrition Research Lab at UNCG, and written informed consent was obtained. A new breast pump was opened and washed by the participant, then allowed to air dry. While the breast pump was drying, the following data were collected: maternal age, height, weight, and recent illness; infant age and recent illness; time, duration, and breast used for the most recent HM expression (via pump and/or feeding); and total number of HM expressions for the day. Additional dietary information was collected for use in a different study, which included: skin carotenoid levels using a Veggie Meter (617W0045; Longevity Link, Salt Lake City, UT USA); and two dietary assessments, a 24-hour food record with 5-step multiple-pass[36] and Rapid Eating Assessment for Patients (REAP)[37] questionnaire. All data were recorded using a 3-digit anonymous identification code (ID). Next, usage instructions for the breast pump were reviewed and the instructional video was available if requested. Participants were asked to express HM from one or both breasts, with a goal of achieving 90 mL of expressed HM. Duration, breast(s) used, and total volume of the sample were recorded. When the participant finished pumping, the researcher transferred the sample to a covered and labeled 200 mL glass beaker, then returned the pump to the participant.

Fresh HM Sample Handling

Fresh HM was immediately transported to the Perrin Lab at UNCG. Temperature and pH were measured using an Orion Dual Star pH/ISE meter (2115205; Thermo Scientific, Waltham, MA USA) and baseline aliquots of fresh HM were collected for future analysis. Briefly, a preliminary investigation determined that magnetic mixing on a stir plate (11-498-7SH; Fisher Scientific, Bohemia, NY USA) for 5 minutes at a moderate speed (4–5 on a scale of 1–10, depending on volume) was necessary to obtain representative aliquots. After 5 minutes, mixing continued while 500-µL aliquots were pipetted into 1.5 mL microtubes and stored at -20°C. Aliquot volume was based on the minimum amounts necessary for sample analysis, to ensure that the majority of HM was available for the thawing/decanting treatments. The remaining HM was equally divided between two commercial storage bags (NUK Seal 'N Go; Newell Brands, Hoboken, NJ USA). Bags were labeled with participant ID, date, and treatment (described in the following section), and filled using a Drummond pipet aid (7780A20; Thomas Scientific, Swedesboro, NJ USA) and 25 mL serological pipet (1163Y23; Thomas Scientific, Swedesboro, NJ USA). Equal volumes of HM were dispensed into the two bags in an alternating and incremental manner (matching volumes up to 25 mL, depending on total volume). For example, using a starting volume of 112 mL: First, 25 mL was taken up into the pipet and expelled back into the sample beaker — this was done to coat the serological pipet and prevent unequal amounts of fat in the bagged samples. Next, 25 mL was pipetted into one bag chosen at random (bag A), 25 mL in the other bag of the same ID (bag B), 25 mL in bag A, 25 mL in bag B, 6 mL in bag A, and 6 mL in bag B. The bags were then sealed, frozen (lying flat, -20°C), and stored for about 60 days.

Treatment Groups

For this study, we tested two thaw stages (ice/liquid) and two bag manipulation methods (yes/no). For sample IDs 101–123, each ID was assigned to one thaw stage of either liquid or ice, on an alternating cycle based on chronological order. Within an individual ID (101–123), one sample was assigned to be decanted using bag manipulation (yes) and the other sample was not (no). For bags receiving the manipulation treatment, the decanting process involved pouring the contents of the bag into a 250 mL glass beaker, then folding the bag lengthwise and squeezing between the index and middle fingers 2–3 times to remove any remaining contents from the bag. Bags not subjected to manipulation were discarded after the contents of the bag were poured into a 250 mL glass beaker. For sample IDs 124–140, no bag manipulation was used when decanting thawed samples into a beaker. Within an individual ID (124–140), each sample was assigned a different thaw stage (ice/liquid).

At the end of the storage period for a sample ID, both bags started the thaw process on BPA-free plastic trays (CT101406; Carlisle, Scottsdale, AZ USA) at room temperature (around 20°C) for 30 minutes. Bags in the ice group were then decanted into a 250 mL glass beaker using the bag manipulation treatment assigned, covered, labeled, and kept on a tray in the refrigerator overnight (about 14 hours at 4°C) until processing the following day. Bags in the liquid group were placed on a tray in the refrigerator overnight for additional thawing (about 14 hours at 4°C); then decanted into a 250 mL glass beaker using the bag manipulation treatment assigned, covered, labeled, and returned to the refrigerator until processing. Thaw times were determined by a preliminary investigation using the same storage bags and a range of HM volumes. A researcher wearing a lab coat, gloves, and with hair restrained, opened bags one at a time on a bench top in the following manner: An unopened bag was wiped on both sides with a paper towel, gently pulsed 5 times, cut from the top using stainless steel scissors, and contents were poured from the bag into a 250 mL glass beaker.

Post-Decanted Milk Sample Handling

One beaker (chosen at random) from an ID pair was removed from the refrigerator and mixed on a magnetic stir plate for 5 minutes at a low-to-moderate speed (3–4 on a scale of 1–10, depending on volume). After 5 minutes, mixing continued while 1-mL aliquots were pipetted into 1.5 mL microtubes and stored at -20°C. The other beaker in the ID pair was then mixed and aliquoted using the same method. Temperature and pH were recorded.

Sample Analysis

For all analyses, aliquots with the same ID were tested as a set on the same day by the same researcher.

Fat was measured using a modified Mojonnier ether extraction method, developed by Choi et al.[38] This method was found to be precise (CV = 1.7%) and have a close correlation (R^2 = 0.999) with reference values analyzed by two accredited laboratories in Canada.[38] For analysis, 1 mL HM samples were thawed for 15–20 minutes at 40°C using a Digital Heating Cooling Drybath (88880029; Thermo Fisher Scientific, Waltham, MA USA), vortexed for 15 seconds, and pipetted into at 15 mL tube. The 15 mL tube containing the HM was weighed on a digital scale, and mass of the HM (M1) was calculated by subtracting the mass of the empty 15 mL tube. Next, 0.5 mL of ammonium hydroxide and 5 drops of phenolphthalein solution were added to the 15 mL tube. To extract fat from the HM, 1.0 mL ethanol, 2.5 mL ethyl ether, and 2.5 mL petroleum ether are added to the 15 mL tube. Tubes were centrifuged at 4000 g for 3 minutes. When complete, two colored phases were visible in the sample — a clear non-polar phase (from the ether) and a violet aqueous phase (from the ammonium hydroxide / phenolphthalein solution). The clear phase was pipetted on to a decanting dish and placed under a fume hood. The extraction process was performed twice more on the violet aqueous phase by adding 1 mL ethanol, 1.5 mL ethyl ether, and 1.5 mL petroleum ether to the 15 mL tube; then repeating the centrifuging and extracting procedure. The extraction dish was kept under the fume hood for 25 minutes to allow the ether to evaporate, dried at 100°C for 30 minutes in a gravity convection oven, and cooled in a desiccator for 10 minutes. The extraction dish was weighed on a digital scale, and mass of the dried and cooled HM (M2) was calculated by subtracting the mass of the empty decanting dish. One blank sample (using 1 mL water instead of 1 mL milk) was also subjected to the same procedure (M3). Fat in grams (g) was calculated by subtracting the mass of the dried HM solid (M2) by the mass of the dried blank solid (M3), and dividing by the mass of the HM sample (M1).[38] Fat (g) was expressed in terms of volume (g/dL) using the established density of HM, 1.03 g/mL.[39]

Total aerobic bacteria were measured in duplicate using a plate enumeration method. A 0.1% peptone water solution was created using tryptic soy broth (22092; Sigma-Aldrich, St. Louis, MO USA), sterilized in an autoclave, then used to dilute HM samples. Preliminary testing determined that initial samples should be plated using dilution factors of 1:1 (undiluted), 1:10 and 1:100; and post-decanted samples should be plated using a dilution factor of 1:1 and 1:10. For analysis, 1 mL HM samples were thawed for 10 minutes at 20°C using a drybath, then vortexed 10 seconds before pipetting the amount needed for the dilution into a new 1.5 mL microtube. Under a hood, diluted milk samples were vortexed for 10 seconds, and 1 mL of HM sample was pipetted on to a Petrifilm Total Aerobic count plate (70200572124; 3M, St. Paul, MN USA). For each set of HM samples processed, one control plate was created using 1 mL sterile peptone water. Plates were incubated at 35°C for 48 hours, then the number of colony forming units (CFU; visible as red dots) was counted via magnifier and handheld counter. The dilution factor selected per plate was determined per manufacturer's instructions as the dilution factor with the greatest number of colonies < 250 per plate. Total CFU in a 1 mL sample were calculated by multiplying the number of colonies by the chosen dilution factor.

Statistical Analysis

Statistical analysis was conducted using R software (version 3.5.2; R Foundation for Statistical Computing, Vienna, Austria). Paired t-tests were used to evaluate the difference in fat between the two thaw stage groups and between the two bag manipulation groups. Results for bacteria (CFU/mL) were log transformed to achieve a normal distribution, and are reported as logCFU. Repeated measures ANOVA and post hoc Tukey HSD comparison was used to evaluate the difference in logCFU between fresh HM and the two post-decanted (thawed) samples.

Results

Fresh HM samples were obtained from 40 women. Mean sample volume was 108 mL and ranged from 25 to 200 mL. Baseline data on fat content of fresh milk was not available due to changes in our laboratory protocol for measuring fat. The mean fat content of all post-thaw samples was 3.24 g/dL and ranged from 0.75 to 7.38 g/dL.

Impact of Thaw Stage

The difference in the mean fat content between samples thawed to a liquid versus ice stage before decanting was 0.10 g/dL and was approaching statistical significance ($n = 17$, $p = 0.07$).

Impact of Bag Manipulation

Fat: In the ice thaw stage, we did not observe any differences in fat content between bags decanted with manipulation and bags decanted without manipulation (n = 11, p = 0.47). In the liquid thaw stage, samples decanted with bag manipulation retained an average of 0.13 g/dL more fat than samples decanted without bag manipulation (Figure 7; n = 12, p = 0.005).

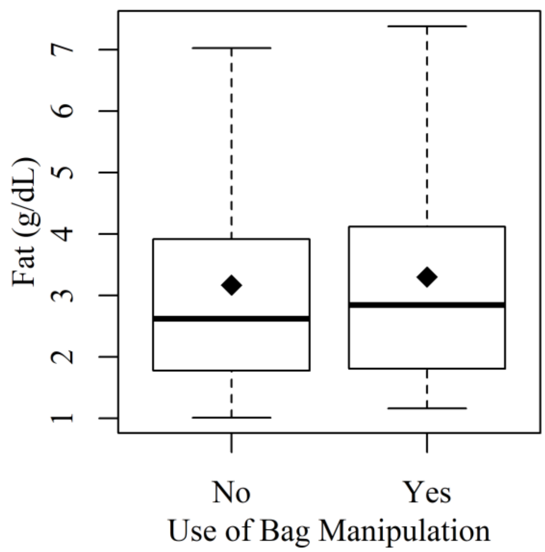

Figure 7. Distribution of Fat by Use of Bag Manipulation for Milk Thawed to a Liquid State. Diamonds (♦) represents mean values; rectangles represent Quartile 1 to Quartile 3; solid lines represent median. Means are significantly different (n = 12; paired t-test, p = 0.005).

Bacteria: Mean logCFU was significantly different between fresh HM and the two thawed/decanted samples (Figure 8; n = 23, p < 0.001). The difference in logCFU between fresh HM and thawed HM decanted with bag manipulation was significant (mean logCFU = 3.5 for fresh, 1.6 for thawed and manipulated; p < 0.001). The difference in logCFU between fresh HM and thawed HM decanted without bag manipulation was significant (mean logCFU = 3.5 for fresh, 1.3 for thawed and not manipulated; $p < 0.001$). We did not observe a difference in mean logCFU between the two bag manipulation treatment groups (mean logCFU = 1.6 for thawed and manipulated, 1.3 for thawed and not manipulated; $p = 0.49$). No bacterial growth was observed on any of the control plates.

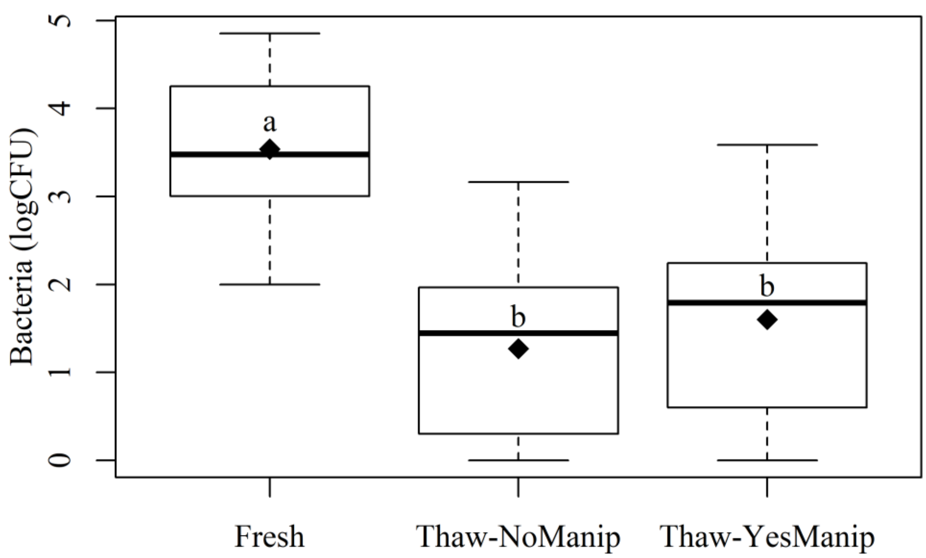

Figure 8. Distribution of Bacteria Pre– and Post–Treatment. Fresh — raw milk; Thaw-NoManip — milk thawed and decanted without bag manipulation; Thaw-YesManip — milk thawed and decanted with bag manipulation. Diamonds (♦) represents mean values; rectangles represent Quartile 1 to Quartile 3; solid lines represent median. Means not sharing the same letter (a, b) are significantly different (n = 23; ANOVA with Tukey HSD, $p < 0.001$).

Discussion

In this novel study that explored how milk banking practices influence fat retention in DHM production, we found that bag manipulation can significantly increase fat recovery when thawing human milk to a liquid state and decanting from plastic storage bags.

Impact of Thaw Stage

We did not observe a significant difference in fat retention between the liquid and ice thaw states; however, the difference was approaching statistical significance and warrants further investigation in a larger study. While other studies have compared fat retention using different methods of thawing, our study was the first to investigate how the degree of thaw when removing HM from a storage bag — ice or liquid — impacts fat retention. A study by Chan et al[30] measured fat loss in HM samples (n=17, 40–100 mL each) thawed to completely liquid using four different methods, and found that fat loss was similar when thawing in a refrigerator (up to 46 hours at 4ºC) or at room temperature (under 4.5 hours at 20ºC), and significant fat loss occurred when HM was thawed using heat (via microwave or 40ºC water bath; $p \leq 0.01$). Thatrimontrichai et al[31] also reported greater losses when thawing 60 mL samples ($n = 90$) at 37ºC for 30 minutes, compared to 4ºC for 24 hours ($p = 0.02$). Neither study reported the temperature of the HM during thawing, so it is unknown if the non-refrigerated thawing methods resulted in HM above 7.2ºC (per HMBANA guidelines).[32] Conversely, Handa et al[34] did not observe a reduction in fat content when 100 mL frozen HM ($n = 40$) was thawed using a combination of 10 minutes at 37ºC (either water bath or dry bath) followed by 24 hours in the refrigerator. However, the temperature of the HM samples reached 20–30ºC, which exceeds HMBANA guidelines. Additionally, none of the aforementioned studies used plastic storage bags.

Impact of Bag Manipulation

We did not observe a difference in fat retention when bag manipulation was used for decanting HM in an ice state, but bag manipulation increased fat retention when decanting HM in a liquid state. We did observe a decrease in total aerobic bacteria after fresh HM samples had been frozen, thawed, and decanted with and without bag manipulation, but we did not observe a difference in total aerobic bacteria between samples decanted with and without bag manipulation. While other studies have compared fat retention and bacterial content of thawed HM using different containers, our study was the first to investigate how the method for removing HM from a storage bag — with and without manipulation — impacts fat retention.

Fat: A study by Janjindamai et al[40] compared pre– and post–thaw measurements for fat content of HM stored in a hard plastic container and soft plastic bag. Fresh HM (n = 90, 80 mL each) was equally divided between four containers — two of each type, 20 mL per container. One of each container type was used for baseline measurements, and the other two containers were frozen for 30 days at -20ºC. Thawing was done in a shaking water bath at 37ºC for 30 minutes, which is outside of the temperature range for thawing human milk in a milk bank setting. Fat loss was not significantly different between the two container types ($p = 0.53$), but was significant compared to fresh HM (about 0.30 g/dL, $p < 0.001$). However, the aliquots used for testing were removed directly from the storage containers, thus the results may not represent fat loss during the decanting process in a milk bank where milk is completely removed from storage containers.[40]

Bacteria: Our results are similar to those in a study by Ahrabi et al,[21] who measured changes in raw HM samples (n = 40) over 9 months of storage at -20ºC. Total aerobic bacteria were quantified via plate enumeration, and decreased rapidly in the first month from about 1.2×10^5 CFU/mL to about 0.7×10^5 CFU/mL ($p < 0.001$), and fell to almost zero after 3 months. The concentrations of two bioactive compounds (lactoferrin and IgA) were not significantly different over the 9-month period ($p > 0.05$). The authors conclude that the decrease in bacteria is likely related to the antimicrobial properties of bioactive compounds remaining active during frozen storage,[21] which is consistent with the literature.[41]

Multiple strategies to reduce bacterial contamination in DHM were reviewed in a paper by Froh et al,[42] which concluded that bacterial contamination was most likely to occur at the donor-level during collection, but contamination could be decreased through donor education; and that milk bank processing methods outlined in the HMBANA guidelines were effective in reducing bacterial contamination at the milk-bank level. For dispensing raw DHM, HMBANA guidelines set a cutoff level of $\leq 10^4$ CFU/mL for non-pathogenic bacteria (e.g. *Staphylococcus epidermis* and other bacteria commonly found on the skin), but do not allow any level of pathogenic bacteria.[32]

Strengths and Limitations

A major strength of our study was the use of fresh HM in order to best represent a milk banking scenario and capture the physical changes that occur during storage in a commercially available container. Each sample was collected on-site using a new pump of the same make/model to reduce possible contamination from home breast pumps.[42,43] Also, one researcher collected and processed all fresh HM samples.

There were several limitations to our study. Volumes of donations ranged from 25 mL to 200 mL, and fat concentrations in the milk ranged from under 1 g/dL to over 7 g/dL, which produced different degrees of thaw and may have confounded some findings. We were not able to quantify absolute loss of fat between fresh and post-thawed samples due to changes in our fat assay protocol. This was a small study with 11–17 samples used for testing each main effect. Our findings of less fat loss in liquid versus ice thaw that was approaching statistical significance warrants further research in a larger study. Finally, we were not able to replicate all the conditions seen in milk banks — for example, the use of reusable storage containers (instead of bags) is gaining popularity.[44]

Conclusion

For human milk frozen in a plastic storage bag, fat retention may be improved without increasing bacterial contamination by using bag manipulation during decanting when thawing to a liquid state.

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CHAPTER V

WITHIN-POOL NUTRIENT VARIABILITY IN BOTTLED DONOR HUMAN MILK BY METHODS OF MIXING DURING BOTTLING

Abstract

Background: The influence of milk banking processes on nutrient variability in donor human milk (DHM) is largely unknown. Previous studies have measured nutrient variability between pools of DHM, but within-pool nutrient variability (between bottles from the same pool) has yet to be elucidated. *Research Aim*: To gain a better understanding of the effect of different mixing methods during bottling on the distribution of fat, protein, immunoglobulin A (IgA), and lysozyme in bottled, raw DHM. *Methods*: DHM pools were created in a laboratory setting according to HMBANA guidelines and assigned a mixing condition (mixing method during bottling, container material, and hold time). Four mixing protocols using glass pooling containers were tested: Control (no mixing during bottling); Manual-A (manually swirl after pouring 3 bottles); Manual-B (manually swirl after pouring every bottle); and Mechanical-G (continuous mechanical stirring with a magnet). As secondary objectives, we evaluated the impact of plastic pooling containers with mechanical mixing (Mechanical-P), and 24-hour refrigerated delay before bottling with manual mixing (Manual-A24). ANOVA, t-tests, and linear regression were used to compare between– and within–treatment effects. *Results*: There was no difference in nutrient variability between Manual-A, Manual-B, and Mechanical-G, and all were significantly different from the Control group (p <

0.001). There was no difference between a glass or plastic pooling container when mechanical mixing was used ($p > 0.15$). Holding a pool in the refrigerator for 24 hours before bottling created significantly greater variability in fat than pools held for 1 hour ($p < 0.01$). *Conclusion*: Manual and mechanical mixing of 1700 mL DHM pools produces similar fat and protein variability when DHM is pooled and bottled on the same day. Pooling container (glass versus plastic) did not significantly impact nutrient variability within pools. When DHM is pooled on one day and bottled on a subsequent day, more mixing is needed to reduce variability of fat.

Introduction

When a mother's own milk is not available, experts — such as the American Academy of Pediatrics, Academy of Nutrition and Dietetics, and World Health Organization — encourage the use of donor human milk (DHM) for preterm infants in neonatal intensive care units (NICU).[1–5] Before arriving at a NICU, DHM is processed by a milk bank to create a safe product. Typical processing steps in milk banks include: screening donors; receiving and storing donations; thawing, decanting, pooling and bottling milk; and pasteurizing and testing finished products prior to distribution.[6]

For milk banks in the Human Milk Banking Association of North America (HMBANA) network, DHM is processed per evidence-based guidelines,[7] although certain recommendations are detailed in a manner that generates individual variation at some steps. For example, the guidelines state that milk should be maintained $\leq 7.2^{\circ}C$ throughout processing, and that containers of pooled milk may be held in a refrigerator ($\leq 4^{\circ}C$) before and after bottling — but no specific hold time is recommended. Similarly, there are no recommendations for how to mix milk during bottling, despite evidence from the dairy industry that undisturbed milk will separate into fat and skim layers,[8] which may result in an uneven distribution of nutrients, especially fat, during bottling.[9] Dairy science research indicates that when milk is held undisturbed in the refrigerator for 24 hours, fat begins to rise and form a top layer within 40 to 50 minutes.[8,10] The majority of separation occurs at a rapid rate during the first hour, then at a slow but continuous rate for the remaining 23 hours.[11] After fat separation occurs, the method of pouring milk into bottles may also influence the distribution of fat. Researchers found that when poured manually from of the top of a container, the fat and the skim layer were able to be separated into two different bottles; but pouring with a device that removed milk from the bottom of a container (e.g. a spigot) created a rolling action that mixed the fat and skim layers together.[9] Little is known about the impact of mixing during bottling on the distribution of nutrients in human milk, but dairy science research suggests that mixing may be important. In addition to the mixing of large pools of DHM during production, individual bottles of DHM are mixed in a clinical setting prior to administering feeds. Ultrasonication of human milk has been used clinically to improve fat delivery and absorption in preterm infants, though there is limited information on how ultrasonication impacts other nutrients in DHM.[12–14]

The purpose of this pilot study was to better understand the effect of different methods for mixing pools of DHM on the distribution of macronutrients and bioactive factors in bottled, raw DHM. The primary objective was to measure the impact of manual and mechanical mixing on the distribution of total fat, total protein, immunoglobulin A

(IgA), and lysozyme in pooled and bottled DHM. The secondary objective included two exploratory analyses to compare the impact of 1) container material in mechanically mixed pools of DHM and 2) hold time in manually mixed pools of DHM. The tertiary objective was to investigate the impact of ultrasonication on individual bottles of DHM. Fat and protein were selected because of their role in supporting preterm infant growth in the NICU,[15–18] and IgA and lysozyme were selected because they confer important immunoprotective benefits to preterm infants.[19–21] We hypothesized that mechanically mixed pools of DHM will have less variation in the distribution of nutrients than manually mixed pools; and the mechanically mixed pool in the glass and plastic containers will have similar variation.[22] We also hypothesized that ultrasonication on a single bottle of DHM will decrease IgA and lysozyme activity.

Materials and Methods

This study was reviewed by the University of North Carolina Greensboro Institutional Review Board and categorized as non-human subject research (protocols 17-0140 and 17-0523). Frozen, raw human milk designated as research-grade was obtained from Mothers' Milk Bank of Florida (Orlando, FL), Mothers' Milk Bank of the Western Great Lakes (Elk Grove Village, IL), and WakeMed Mothers' Milk Bank and Lactation Center (Cary, NC). Our goal was to create bottles of pooled DHM that were similar to those produced by milk banks, thus our pooling and mixing methodologies were modeled after methods observed during an environmental scan we conducted between 12/2016 and 4/2018 of 9 milk banks within the HMBANA network, as well as the HMBANA guidelines.[7] One researcher completed all processing and analyses, and temperature was monitored at multiple points during processing using a digital thermometer (11779725; FisherBrand, Goteborg, Sweden).

Thawing, Decanting, and Pooling

Six pools were created for this study — 4 to test our primary objective of mixing methods, and 1 pool each to assess the secondary objectives of container type and hold time. Each pool contained milk from the same 4 donors with similar ranges of pump dates. Approximately 1700–1900 mL of milk was thawed for each pool. This volume was selected to achieve a pool volume ranging 1600–1800 mL and account for milk that may escape from leaky bags during the thawing process. Frozen storage bags of milk were thawed on BPA-free plastic trays (CT101406; Carlisle, Scottsdale, AZ USA) at room temperature (around 20°C) for 2 hours, then placed in a refrigerator (4°C) for 24 hours. Thawed milk was decanted into the assigned pooling container — either a 2000 mL wide-mouth glass flask or 2000 mL BPA-free plastic beaker (depending on mixing condition). Once all bags had been decanted, the pooling container was manually swirled for 30 seconds until no visible fat layer remained, then covered and placed in the refrigerator for a pre-determined hold time. Pooled milk is often returned to the refrigerator before bottling commences to keep pool temperatures below 7.2°C per HMBANA guidelines.[7]

After the pre-determined hold time, the pooling container was removed from the refrigerator and again manually swirled for 30 seconds until no visible fat layer remained, then returned to the refrigerator for 20 minutes. During this time, one researcher prepared several sterile sample cups (03008-7TN; Starplex Scientific Corp, Cleveland, TN USA) for bottling. The number of cups was determined by the volume of the pool. The cups were labeled with a numeric ID to represent the order of pour within a pool, then placed in numerical order with lids removed, in preparation for bottling. Since the cups were used to simulate the bottling process, they were referred to as bottles.

Mixing During Bottling

The conditions for mixing during bottling (Table 5) were determined based on common methods observed during our environmental scan.

Table 5. Summary of Processing Factors Used for Each Pool.

Treatment Name	**Mixing During Bottling**	**Pooling Container**	**Hold Time at 4ºC**
Control	No mixing	Glass	1 hour
Manual-A	Intervals of manually swirling 2 seconds, then manually pouring 3 bottles	Glass	1 hour
Manual-A24	Intervals of manually swirling 2 seconds, then manually pouring 3 bottles	Glass	24 hours
Manual-B	Intervals of manually swirling 2 seconds, then manually pouring 1 bottle	Glass	1 hour
Mechanical-G	Continuous mechanical stirring with magnet, poured via dispensing pump	Glass	1 hour
Mechanical-P	Continuous mechanical stirring with magnet, poured via dispensing pump	Plastic	1 hour

Note: Hold Time — the amount of time the 2000 mL container of pooled milk was held in the refrigerator prior to commencing bottling.

The four pools for our main objective (Control, Manual-A, Manual-B, and Mechanical-G) were all prepared in a 2000 mL wide-mouth glass flask and held for 1 hour in the refrigerator before bottling commenced using the assigned mixing method. Although not observed during the environmental scan, a mixing protocol that reflected no additional mixing once bottling commenced was used as a control (Control). The amount of mixing increased with each subsequent treatment group. Pools receiving the manual mixing method were held by a researcher with both hands — one at the neck of the flask and the other supporting the base of the flask. Bottling commenced by swirling the flask in a circular motion while counting out-loud for 2 seconds, then manually pouring either 3 bottles (Manual-A) or 1 bottle (Manual-B), visually approximating 90 mL of DHM per bottle. The pool receiving the mechanical mixing method (Mechanical-G) was mixed for 5 minutes on a magnetic stir plate (11-498-7SH; Fisher Scientific, Bohemia, NY USA) at a moderately high speed (8 on a scale of 1–10). After 5 minutes, mixing continued while DHM was bottled using a dispensing pump plus handheld wand attachment (AR77922-32; Argos Technologies, Vernon Hills, IL USA). This device was programmed for 90 mL at a pump speed of 150 rpm, and primed by dispensing the first 3 90-mL pours of DHM back into the pooling container. Speed of mixing decreased as the volume of DHM in the pooling container decreased.

As an exploratory secondary objective, one additional pool was prepared in the same manner as Manual-A, however it was held for 24 hours in the refrigerator before mixing during bottling commenced (Manual-A24); and one additional pool was prepared in the same manner as Mechanical-G, however it was pooled in a 2000 mL BPA-free plastic beaker (Mechanical-P).

Aliquoting Study Samples

Each of the mixing conditions produced 18–20 bottles of DHM, which were stored in a refrigerator for 24 hours. One bottle at a time was removed from the refrigerator for aliquoting. A preliminary investigation determined that magnetic mixing on a stir plate (11-498-7SH; Fisher Scientific, Bohemia, NY USA) for 5 minutes at a moderate speed (4–5 on a scale of 1–10) was necessary to obtain representative aliquots from each bottle. After 5 minutes, mixing continued while 1-mL aliquots were pipetted into 1.5 mL microtubes and stored at -20°C until analysis.

Ultrasonication

We also tested the impact of ultrasonication on individual bottles of DHM, which has been explored as a technique to improve fat delivery to NICU infants during tube feedings.[12–14,23] Our ultrasonication protocol was based on methods from those tube feeding studies, specifically Garcia-Lara et al,[23] who used the same make and model of ultrasonicator that was available in our lab (VCX 130; Sonics and Material, Newtown, CT USA). Immediately after aliquoting, a subset of bottles (n = 37) were removed from the magnetic mixer and the remaining 80 mL of DHM was homogenized with an ultrasonicator. A 12 mm probe was used with 75% amplitude for 120 seconds (based on a rate of 1.5 seconds/mL). Ultrasonicated DHM was aliquoted into 1-mL increments and stored at -20°C until analysis.

Sample Analysis

For all analyses, aliquots from the same treatment group were tested as a set after thawing for 10 minutes at 30°C using a Digital Heating Cooling Drybath (88880029; Thermo Fisher Scientific, Waltham, MA USA).

Fat was measured using the creamatocrit method by Lucas et al, which uses the percentage of cream to calculate the fat content of milk.[24] Meier et al. found creamatocrit to have high intra– and inter–user reliability (all mean differences < 1%), and ability to measure fat content similarly to other common laboratory procedures.[25] In a comparison of three methods for measuring fat, Du et al found creamatocrit to be precise (CV = 3.9%) and have a close correlation ($R^2 = 0.995$) with the gravimetric method (a gold standard reference method), despite under-reporting by 0.3–0.6 g/dL.[26] Although this technique is more operator-dependent than other tests,[27,28] a preliminary investigation illustrated that the researcher was able to achieve consistently low CVs (< 3%) by vortexing the microtubes (as opposed to manual inversions) and using a flatbed centrifuge to create better distinguished fat layers. Specifically, a microtube of DHM was vortexed for 10 seconds at level 5 intensity on a Vortex Genie 2 (12-812; Fisher Scientific, Bohemia, NY USA) prior to filling each capillary tube. The capillary tubes were spun for 10 minutes at 11.2 x 1000 rpm on a Zip-IQ PCV Centrifuge (ZiC-24HD-75T3; LW Scientific, Lawrenceville, GA USA), as determined by Miller et al,[27] then measured using a Creamatocrit Plus (100-146; EKF Diagnostics, Boerne, TX USA). Creamatocrit values were converted to fat (g/dL) with the equation determined by Meier et al[25]: $(3.968 + 5.917 \times \text{creamatocrit}) \div 10$.

Protein was measured via Pierce bicinchoninic acid assay (BCA; 23225; ThermoFisher Scientific, Rockford, IL USA), which has been validated for human milk,[29] although it likely overestimates by as much as 30%.[27] This technique is based on the reduction of Cu^{+2} to Cu^{+1} via biuret reaction, where Cu^{+1} is colorimetrically detected when it reacts with a BCA reagent. A set of samples was thawed, diluted 1:10, and loaded in a 96-well plate with standards made using bovine serum albumin. After adding the BCA working reagent, the plate was incubated for 30 minutes at 35°C, cooled on ice for 10 minutes, then read using a spectrophotometer (7091000/11120570; BioTek, Winooski, VT USA) — the purple-colored product has a strong absorbance at 562 nm, the intensity of which is proportional to protein concentrations ranging from 20 to 2000 µg/mL. Protein (g/dL) was quantified with a standard curve and adjusted using the formula established by Keller and Neville for comparison to Kjeldahl methods.[29]

Bioactive factors were quantified by activity level, using modified versions of the IgA method from Chen,[30] and lysozyme method from Lee and Yang,[31] as described in the thesis papers of both Meng[32] and Wagner-Gillespie.[33] IgA activity was measured using an Enzyme Linked ImmunoSorbent Assay (ELISA), which assessed the ability of IgA to bind to an *Escherichia coli* (*E.coli*) antigen.[30] To complete this assay, *E.coli* antigens were prepared from reference strains (ECOR-3, 8, 10, 34, 39, 49, 51, 59; Michigan State University STEC Center, East Lansing, MI USA), then the antigens were pipetted into a 96-well plate and incubated at room temperature for 12–18 hours to ensure binding to the bottom of plate. The plate was washed three times with a 0.01 M Phosphate Buffered Saline + 0.05% Tween 20 solution (PBST), human milk samples diluted 1:100 with PBST were added, and then the plate was incubated at room temperature for 3 hours to allow IgA to bind to the antigen. After an additional wash series, a horseradish peroxidase-labeled anti-human IgA antibody (A0295; Sigma-Aldrich, St. Louis, MO USA), which binds to IgA in milk, was added. Another wash series was performed, and a 0.05 M Citrate Buffer + 3% Hydrogen Peroxide + 40 mM 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid substrate solution was added to initiate a change in color of the horseradish peroxidase, the intensity of which changes over time and is measured by optical density via spectrophotometer (7091000/11120570; BioTek, Winooski, VT USA) at a wavelength of 405 nm. The plate was read immediately, then once every 2 minutes for a total of 20 minutes, with slow mixing by the machine occurring for 5 seconds before each reading (when available on the plate reader). Activity level of IgA was determined using the change in optical density over time and the linear regression formula from a purified human IgA (I2636; Sigma-Aldrich, St. Louis, MO USA) standard curve.[30]

Lysozyme activity was measured using a method developed by Shugar[34] and adapted to a 96-well plate by Lee and Yang.[31] The method used a Synergy HT plate reader (7091000; BioTek, Winooski, VT USA) to assess turbidity change in a 0.015% suspension of *Micrococcus lysodeikticus* (M3770; Sigma-Aldrich, St. Louis, MO USA), where a decrease in turbidity of 0.001 Å/minute at an absorbance of 450 nm with a pH of 7.0 and a temperature of 25ºC equaled one unit of lysozyme activity.[34] A 66 mM potassium buffer was used to create a 0.015% suspension of *Micrococcus* lysodeikticus, and human milk samples were diluted 1:100 with deionized water. A 96-well plate was loaded with the diluted human milk and bacterial suspension, then read at 450 nm every minute for 7 minutes while temperature was maintained at 25ºC. Lysozyme activity (units/mL) was quantified with the following equation: [(average change in absorption per minute) ÷ (0.001 × 0.025)] × 100; where 0.025 is the volume (mL) of human milk per well and 100 is the dilution factor of the human milk.[31]

Before completing all sample analyses, our Synergy HT plate reader (7091000; BioTek, Winooski, VT USA) was retired and replaced by an Epoch plate reader (11120570; BioTek, Winooski, VT USA), that did not include a plate-mixing option. To account for the plate reader as a confounding variable, the analysis of IgA for mixing method was stratified by plate reader (Reader1/Reader2), and the analysis of IgA between hold times was excluded because the two treatment groups (Manual-A and Manual-A24) were measured by different plate readers. The analysis of lysozyme between hold times was also excluded because Reader2 was not equipped for a temperature-dependent assay, thus lysozyme was not measured in the Manual-A24 group. The change in plate readers did not affect analyses for protein.

Statistical Analysis

Statistical analysis was conducted using R software (version 3.5.2; R Foundation for Statistical Computing, Vienna, Austria). To control for differences in nutrient content between pools of DHM, the absolute percent difference from the mean (%diff; calculated for each sample using the treatment-specific mean for a given nutrient) was used as the outcome variable unit for between-treatments comparisons of mixing methods. Absolute value was used because strong deviations from a mean nutrient content — regardless of directionality — would be undesirable in a milk bank. The within-treatment comparison (pour order) used the ratio of fat content of bottles within a pool to the fat content of the first bottle in the pool, which served as the baseline. This allowed us to compare magnitudes of trends between pools that started with different fat content. For evaluation of the impact of ultrasonication on an individual bottle of milk, actual nutrient values were used as the outcome variables.

For our primary objective, differences between 4 mixing conditions that used glass pooling containers and a 1-hour hold time were assessed with ANOVA and post hoc comparison using Tukey HSD. Linear regression was used to determine if pour order within a mixing condition was a predictor of change in fat content. The influences of pooling container material and hold time were explored using unpaired t-tests. A paired t-test was used to investigate the impact of ultrasonication on the retention of fat, protein, IgA, and lysozyme within a bottle of DHM.

Results

Fat was measured in duplicate, and protein, IgA, and lysozyme were measured in triplicate (average CVs for replicate measures were 1.8%, 2.5%, 4.1%, and 8.3%, respectively). The mean values of the pools created in this study ranged from 2.88 to 4.32 g/dL for fat (n = 6 pools and 114 samples), 1.05 to 1.27 g/dL for protein (n = 6 pools and 114 samples), 0.6 to 1.18 mg/mL for IgA (n = 6 pools and 114 samples), and 33773 to 45747 units/mL for lysozyme (n = 4 pools and 76 samples).

Impact of Mixing During Bottling Between Pools of DHM

Mixing Method: To better isolate the effect of mixing method, only treatments with the same pooling container material (glass) and hold time (1 hour) were used for our primary analysis — Control, Manual-A, Manual-B, and Mechanical-G. Bottles within a pool differed significantly from the pool mean by mixing methods for fat (Figure 9, $p < 0.001$), but not protein ($p = 0.20$) or lysozyme ($p = 0.99$). Specifically, the mean %diff for fat in the Control (9.0%) was significantly larger than the mean %diff for fat in Manual-A (2.3%, $p < 0.001$), Manual-B (2.2%, $p < 0.001$), and Mechanical-G (2.1%, $p < 0.001$), but there was no statistical difference between Manual-A, Manual-B, and Mechanical-G ($p = 0.95$). When stratified by plate reader, mean %diff for IgA was not significantly different between mixing groups measured with Reader1 (Manual-A and Manual-B; $p = 0.21$), nor was it significantly different between mixing groups measured with Reader2 (Control and Mechanical-G; $p = 0.94$).

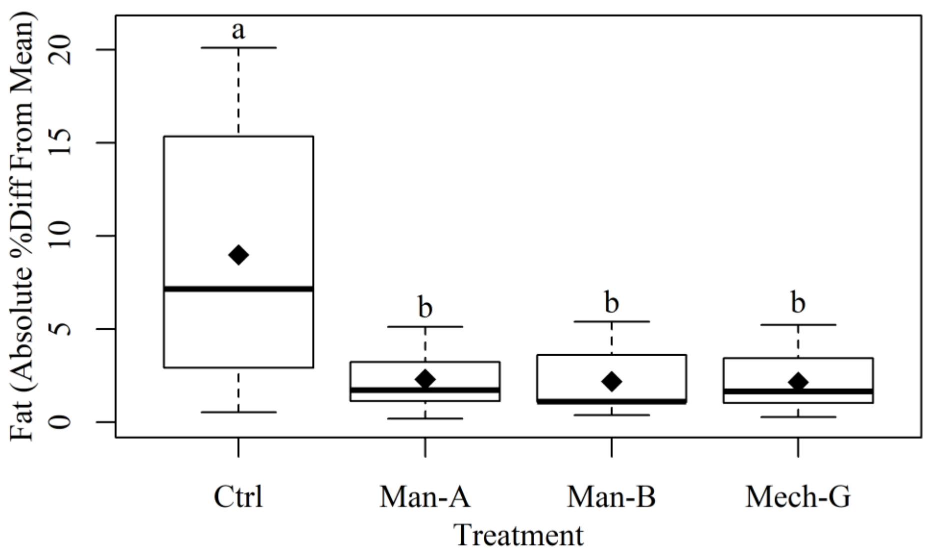

Figure 9. Distribution of Fat by Treatment Group. Diamonds (♦) indicate mean values, rectangles represent Quartile 1 to Quartile 3; solid line represents median. Means not sharing the same letter (a, b) are significantly different (n = 18 for Control, n = 19 for Manual-A, Manual-B, and Mechanical-G; Tukey HSD, $p < 0.001$).

Container Material: When mechanically mixing a pool with a continuous magnetic mixer after a 1-hour refrigerated hold, the mean %diff between the glass pooling container (Mechanical-G) and plastic pooling container (Mechanical-P) was not significantly different for any nutrient (p = 0.65 for fat, 0.17 for protein, 0.84 for IgA, and 0.32 for lysozyme).

Hold Time: For pools of milk in glass containers that used the same mixing during bottling method, the 1-hour hold group (Manual-A) had a lower mean %diff for fat (Figure 10; 2.3% vs 5.1%, respectively; p = 0.002), compared to the 24-hour hold group (Manual-A24). Mean %diff for protein was not significantly different between the two groups (p = 0.56).

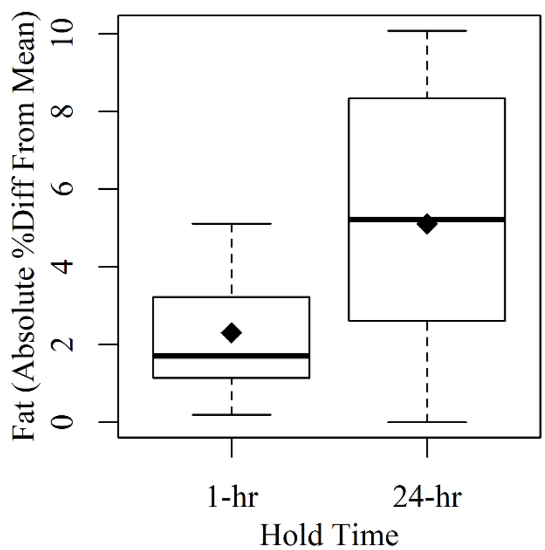

Figure 10. Distribution of Fat by Hold Time. Diamonds (♦) indicate mean values, rectangles represent Quartile 1 to Quartile 3; solid line represents median. Means are significantly different (n = 19 for 1-hour; n = 20 for 24-hour; unpaired t-test, p = 0.002).

Pour Order: Pour order of bottles was not a significant predictor of the change in fat for Manual-A24, Manual-B, or Mechanical-P mixed pools (all p > 0.05). For Control, pour order explained 59.0% of the model for predicting fat (Figure 11) — the model's intercept was at 1.23 (SE = 0.04, 95% CI [1.15, 1.31]) and the effect of pour order was significant (B = -0.02 ± 0.004, 95% CI [-0.02, -0.01], $p < 0.001$). For Manual-A, the pour order explained 54.2% of the model for predicting fat (Figure 11) — the model's intercept was at 1.00 (SE = 0.01, 95% CI [0.98, 1.02]) and the effect of pour order was significant (B = -0.004 ± 0.001, 95% CI [-0.01, -0.002], $p < 0.001$). For Mechanical-G, pour order explained 20.9% of the model for fat (Figure 11) — the model's intercept was at .99 (SE = 0.01, 95% CI [0.96, 1.01]) and the effect of pour order was significant (B = -0.002 ± 0.001, 95% CI [-0.004, -0.00001], $p = 0.049$).

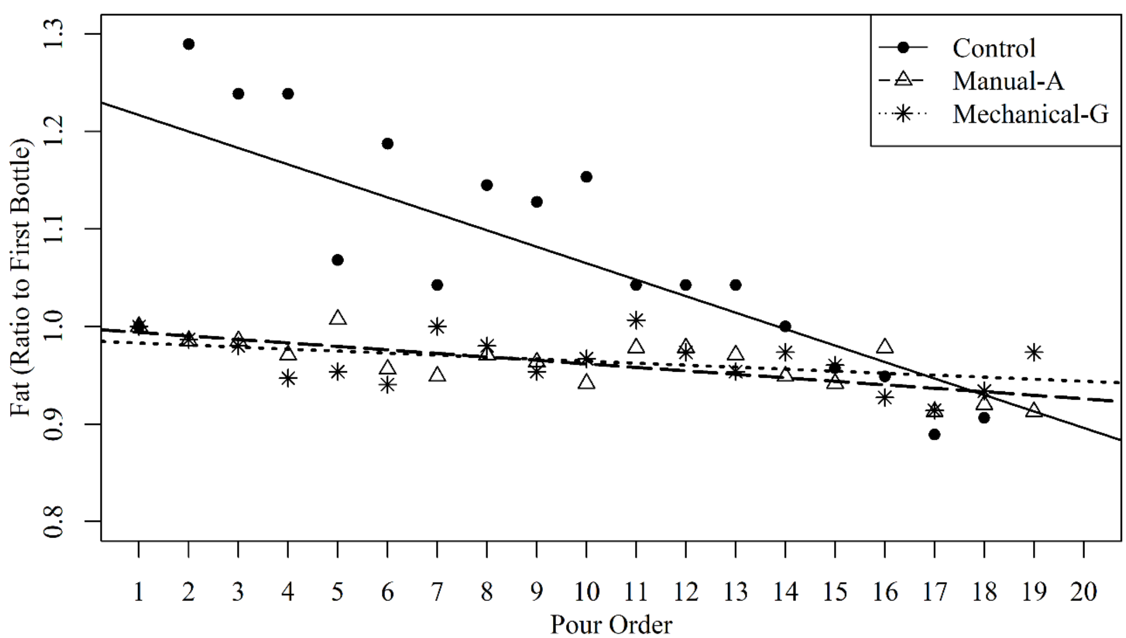

Figure 11. Fat Content Per Bottle (Expressed as a Ratio to the First Bottle) by Pour Order. Lines represent regression model for Control (n = 18, $R^2 = 0.59$, $p < 0.001$), Manual-A (n = 19, $R^2 = 0.54$, $p < 0.001$), and Mechanical-G (n = 19, $R^2 = 0.21$, $p = 0.049$).

Impact of Ultrasonication Within a Bottle of Pooled DHM

The mean temperature of the pre-ultrasonicated samples was 6.2ºC (ranged 5.0 to 8.0ºC), and the mean temperature of the post-ultrasonicated samples was 21.2ºC (ranged 18.7 to 25.1ºC). Compared to the mean nutrient content of the pre-ultrasonicated samples, the mean nutrient content of the post-ultrasonicated samples was 0.64 g/dL lower for fat (95% CI [0.58, 0.69], $p < 0.001$), 0.11 g/dL higher for protein (95% CI [0.09, 0.14], $p < 0.001$), 0.08 mg/mL lower for IgA (95% CI [0.05, 0.11], $p < 0.001$), and 6406 units/mL lower for lysozyme (95% CI [3269, 9544], $p < 0.001$). Findings are illustrated in Figure 12. These differences equate to a 16% decrease in mean fat, an 11% increase in mean protein, a 10% decrease in mean IgA, and a 16% decrease in mean lysozyme.

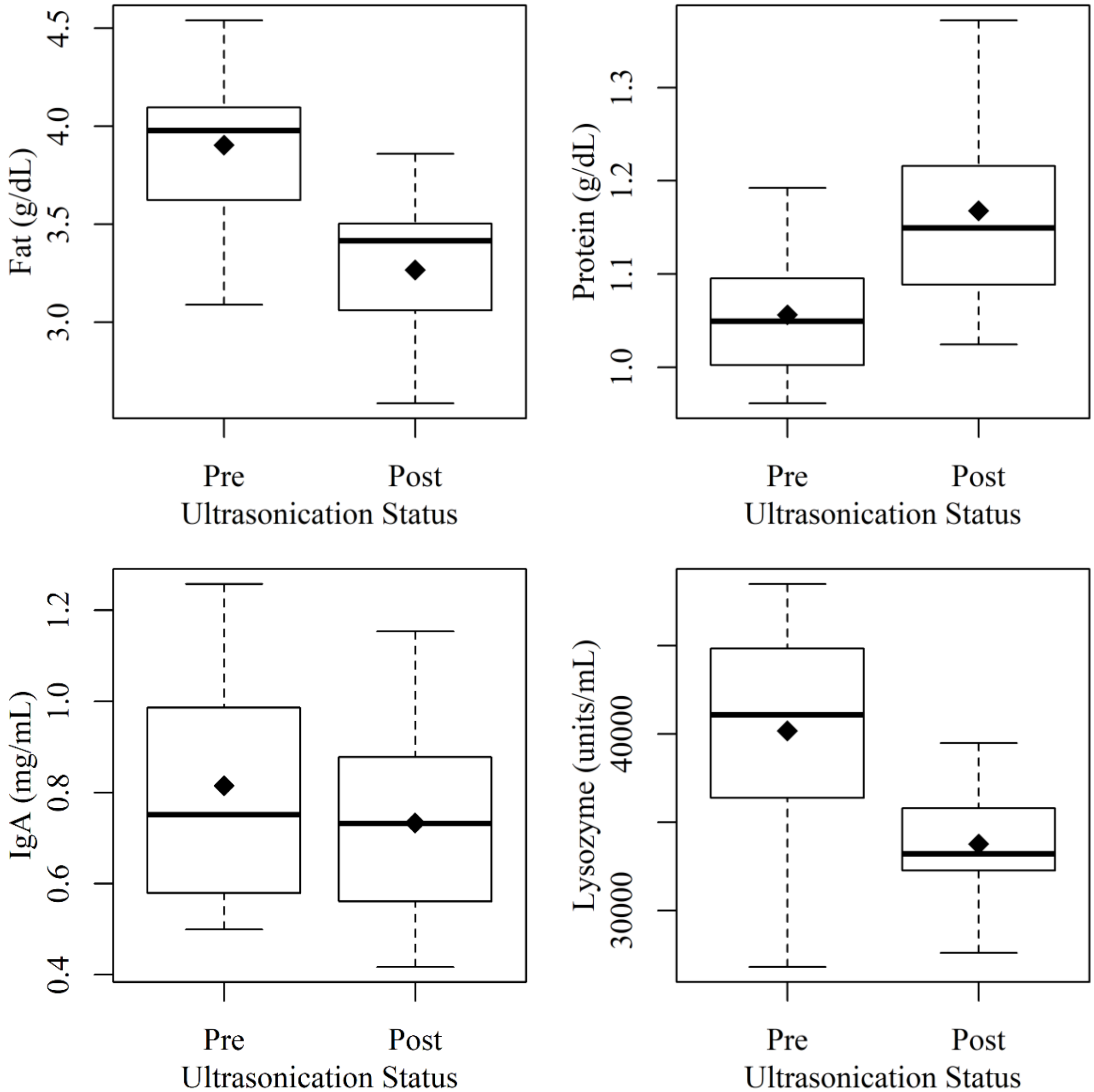

Figure 12. Distribution of Nutrients Pre– and Post–Ultrasonication. Includes fat (top left, n = 37), protein (top right, n = 37), IgA (bottom left, n = 37), and lysozyme (bottom right, n = 19). Diamonds (♦) indicate mean values, rectangles represent Quartile 1 to Quartile 3; solid line represents median. Means are significantly different (paired t-test, $p < 0.001$).

Discussion

Our research fills an important gap in the literature regarding evidence-based recommendations for how DHM should be mixed during bottling to ensure even distribution of nutrients between bottles in a pool. Using pools of approximately 1700 mL of milk, we found that as long as some degree of mixing occurred during bottling, the variability of fat, protein, and lysozyme was not significantly different between manual and mechanical mixing methods for pools of DHM that were pooled in glass containers then bottled after a 1-hour hold time in the refrigerator. No differences in nutrient variability were observed between glass and plastic pooling containers when used with mechanical mixing after a 1-hour hold time. However, when pools of DHM sit overnight in the refrigerator prior to bottling, additional manual mixing is required to reduce between-bottle variability of fat. We also found that under some mixing scenarios, the distribution of fat between bottles in a pool may be predicted by pour order. Our study was the first to investigate within-pool nutrient variability, by measuring nutrient content of bottles from the same pool, in order to understand how much variability is generated by milk banking processes.

Impact of Mixing During Bottling Between Pools of DHM

Mixing Method: The variability of fat was different between mixing methods (of the same pooling container material and hold time before bottling). The no mixing group (Control) had the largest spread of variation, with absolute differences from the mean (%diff) ranging 0.5 to 20.1%. For all other groups (Manual-A, Manual-B, and Mechanical-G), the highest variation for fat was around 5%. To put this into perspective, a 20.1% variation between bottles in a pool of DHM with a fat content of 3.5 g/dL would yield bottles ranging 2.8 to 4.2 g/dL fat, which equates to a difference of up to 3.7 kcal/oz.

During our environmental scan of the field, we observed all milk banks doing some degree of mixing. When the no mixing group was omitted from analyses, fat variability was no longer significantly different between mixing groups ($p = 0.95$), indicating that manual and mechanical mixing methods have similar fat variabilities, which is in opposition to the results of our previous study,[35] where we observed an inverse relationship between fat variability and mechanical mixing compared to manual mixing ($s^2 = 0.45$ for manual and 0.22 for mechanical, $p < 0.001$). However, our previous study was an observational study that did not look at within-pool variations, therefore it was not designed to assess causality of mixing methods. There are no other published studies that compare nutrient variability between methods of mixing pools of DHM in the milk bank setting. Research from the dairy industry has been conducted with large volumes of milk and under multiple mixing conditions. For example, Cunningham[36] found that a duration of 2 to 3 minutes was sufficient to evenly distribute fat under a variety of mechanical mixing conditions for volumes under 2000 L. Duration was influenced by the size of the impeller (e.g. magnet or wand) relative to the length-to-width ratio of the container, as well as location of the impeller in the container. For example, a wider container would need either a larger impeller or a longer duration; and an impeller placed near the bottom of a container would necessitate a longer duration. However, this was a simulated study that used a 21ºC oil and water mixture in place of milk, with scaled-down models of containers and impellers, thus the results may not translate to a milk banking scenario.[36] Additionally, no manual mixing techniques were assessed in their study.

Container Material: We did not observe significant differences in nutrient variability (fat, protein, IgA, or lysozyme) between the glass pooling container and plastic pooling container when mechanical mixing with a magnet was used after a 1-hour hold.

Most of the research about container materials are not within the lens of milk banking (where large containers are used to hold large pools of DHM), and instead assessed smaller containers that were used to hold single samples of milk. For example, a 1981 study by Goldblum et al[37] compared the impact of glass and plastic containers on the loss of bioactive compounds in milk stored up to 24 hours at 4ºC. The authors concluded that no container was superior regarding the loss of bioactive compounds, yet recommended plastic containers over glass containers due to ease of handing (e.g. glass may break, bags may spill).[37] Williamson and Murti[38] also compared containers materials held at 4ºC, except only for a duration of 7 hours and using glass and stainless steel. The authors conclude that at the microscopic level, steel containers are not as smooth as glass, and certain compounds in milk (e.g. immunoglobulins and lysozyme) may more readily adhere to the walls of steel containers.[38] These results from Williamson and Murti,[38] combined with the reported increase in popularity of steel containers in milk banking,[35] warrants further investigation about the impact of steel containers on nutrient variability in the milk bank setting.

Hold Time: We found that variability of fat, but not protein, was significantly different between a pool of DHM held at 4ºC for 24 hours, compared to a pool held at 4ºC for 1 hour, when manual mixing was used. The highest variation for fat in the 24-hr hold pool was 10.1%, compared to 5.1% in the 1-hr hold pool. To put this into perspective, a 10.1% variation between bottles in a pool of DHM with a fat content of 3.5 g/dL equates to bottles with a difference of up to 1.9 kcal/oz.

A study on dairy milk by Servello et al[10] investigated the minimum duration of mixing necessary to evenly distribute fat (defined as a variation of < 0.1% wt/wt between samples) after milk had begun to separate and form a cream layer. The researchers tested tanks of milk at dairy farms in Ontario, Canada, where regulations required tanks to be mixed intermittently throughout the day. A mechanical impeller was used to mix the milk — intervals varied, but most occurred once per hour for at least 5 minutes, and the authors hypothesized that this duration could be reduced to 2 minutes. To test their hypothesis, intermittent mixing was ceased, and milk was held at 4ºC for up to 3 hours, then mixing resumed and a 30 mL sample was removed from the tank every 10 to 30 seconds. The average volume of milk in a tank was about 2000 L and average mixing speed was 35 rpm, although characteristics of the impeller were not stated. Fat distribution was within the defined parameters after 20 to 34 seconds in milk held for 1 hour, and after 56.5 seconds in milk held for 3 hours. The authors concluded that 2 minutes of mixing with an impeller was sufficient to evenly distribute fat for over 99% of tanks in Ontario. However, the results apply to milk that had been mixed intermittently throughout the day, and may not be applicable to unmixed milk, e.g. a container of pooled DHM held up to 24 hours in a refrigerator prior to bottling. The 24-hour hold pool was included in our study to represent conditions observed during the environmental scan, where some milk banks do not bottle on the same day the DHM was pooled. We found that pools of DHM that had been held for 24-hour were not adequately mixed after swirling manually for 30 seconds, compared to pools that had been held for 1-hour. The impact of mechanical mixing on pools held for 24-hour remains unknown and is an important area for future research.

Pour Order: The findings indicate that under some mixing scenarios (Control, Manual-A, and Mechanical-G), there was a linear relationship between pour order and fat distribution of bottles in a pool of DHM. All significant findings had a negative slope, indicating that fat content decreased as more bottles were poured. However, the negative slopes for Manual-A and Mechanical-G visually appear to be near-flat lines, and only represent a 0.4% and 0.2% decline in fat per bottle, respectively; compared to a 2.0% decline in fat per bottle for the Control group. To put this into perspective, quality control standards used by the United States Department of Agriculture allow for a 0.8% difference in fat between tanks of bovine milk.[39] Our results may not translate to larger pool volumes or other bottle volumes, and there are no published studies that investigate the impact of pour order on nutrient variability in pooled DHM. If pour order would be influential in larger batches of milk remains unknown.

Impact of Ultrasonication Within a Bottle of Pooled DHM

We found that ultrasonication of raw human milk in volumes of about 80 mL resulted in significant changes of nutrient composition. Compared to the average nutrient content of pre-ultrasonicated samples, the average nutrient content of post-ultrasonicated samples was 16% lower in fat and 11% higher in protein, and the average activity levels were 10% lower in IgA and 16% lower in lysozyme.

However, the results for fat and protein may be more nuanced when considering the structural changes caused by ultrasonic waves. The recorded decrease in fat may be the result of compacted fat droplets and not actual fat loss. Fat is packaged into membrane-encased structures called milk fat globules (MFG), which consist of triglycerides, phospholipids, sterols, and free fatty acids.[16] Ultrasonication can disrupt the membrane, making triglycerides in the MFG more susceptible to lipolysis and increasing the amount of free fatty acids.[40] The structure of free fatty acids allows these molecules to tightly compact, and may result in some fat measurement methods (e.g. creamatocrit) to falsely indicate fat loss.[28] The recorded increase in protein may also be due to both structural changes and the assay used to quantify protein content. Research from the dairy industry suggests that ultrasonication can disrupt milk protein structure,[41] which would allow for more peptides (previously unavailable due to the folded structure of the protein) to participate in the reaction. The BCA method is a colorimetric assay based on the relationship between color intensity and the number of peptide bonds, so a disruption in the structure of the protein could allow for more peptide bonds to be available to react with copper, resulting in a more intense color and thus indicating a higher protein content.[42]

The assays we used to measure IgA and lysozyme were based on activity level (not content), thus the reported decrease likely represents a true loss, which could result in decreased immune protein for preterm infants. A study by Czank et al[43] also assessed the impact of ultrasonication on bioactive compounds in milk. Samples of milk were ultrasonicated with a 12 mm probe, at a rate of 5 seconds/mL and 70% amplitude. We used the same size probe, but for a shorter duration (1.5 seconds/mL) and slightly higher amplitude (75%). Their results show a loss of both IgA and lysozyme content (not activity) at rates (λ) of 3.7% (R^2=0.96) and 4.5% (R^2=0.89) respectively.[43] Using the exponential regression equation from the study (% retained = $100e^{-\lambda*time}$), we would expect a retention of 93% IgA content and 91% lysozyme content with our rate of 1.5 seconds/mL. Actual retention was 90% for IgA and 84% for lysozyme.

Two major limitation of previous studies are 1) inconsistent definitions of homogenization, and 2) a lack of detail regarding the ultrasonication device — specifically the attachments and settings used — which poses difficulty for replication and creation of a protocol. For example, Martinez et al[12] considered milk to be homogenized when the average diameter of MFGs was < 1.5µm, which was achieved using a rate of 4 seconds/mL at an intensity of 4 (0–10 scale); but this study did not assess nutrients pre– and post–ultrasonication. Czank et al[43] (discussed above) considered milk to be homogenized when the mean particle size reached 0.6 µm (all particles, not just MFG), which was achieved using a rate of 5 seconds/mL at 70% amplitude. Thomaz et al[13] used a rate of 6 seconds/mL, Garcia-Lara et al[23] a rate of 1.5 seconds/mL at 75% amplitude, and de Oliveira et al[14] used 3 intervals of indirect ultrasonication, each for 5 minutes with 30 seconds of rest. The objectives of aforementioned studies were about ultrasonication relative to gastric tubes and digestion, so pre– and post– nutrient measurements were not reported.

It should also be noted that we found ultrasonication increased the temperature of DHM by an average of 15.1ºC, and all post-ultrasonicated samples exceeded

HMBANA's 7.2ºC limit for temperature during processing.[7] At the time of the environmental scan, no HMBANA banks were using ultrasonicators to homogenize pools of DHM during processing, but ultrasonication has been explored as an alternative method for pasteurizing DHM.[43]

Strengths and Limitations

A major strength of our study was constructing our methods from real-world practices we witnessed during an environmental scan of 9 milk banks. The experimental design of our study allowed for the control of several aspects involved in the mixing during bottling step of DHM processing, thereby allowing us to gain more knowledge about the effects of individual processing variables on nutrient variability in bottled, raw DHM including mixing protocol, pooling container, and pool hold time. The nutrient concentrations in our pools of DHM were similar to nutrient concentrations of pooled DHM in the field; and measurements of those nutrients (fat, protein, IgA, and lysozyme) were done by one researcher, with good CVs, and using established methods. To ensure our samples were sufficiently mixed, we conducted a preliminary investigation to determine the method, duration, and speed of mixing necessary to obtain a representative aliquot, given concerns that inconsistent results from previous studies regarding the fat content in human milk were due to insufficient mixing.[44]

While our treatment groups did represent several iterations of the mixing during bottling variables, we were not able to include all possibilities. For example, stand mixers with different shaped wands (agitation method) and stainless steel (container material) were not represented in our study. Also, we did not test all the pooling volumes and containers observed in the field, including large 5-gallon buckets, or when a single pool was divided across multiple 2000 mL flasks. For any of the variables, a lack of significant results does not infer that there is no effect.

Conclusion

Manual mixing and mechanical mixing of 1700 mL DHM pools produced similar fat and protein variability when DHM was pooled and bottled after a 1-hour refrigerated hold time. Pooling container (glass versus plastic) did not significantly impact nutrient variability within pools. When DHM was pooled on one day and bottled on a subsequent day, more mixing was needed to reduce variability of fat. The findings of our study were based on fat, protein, IgA, and lysozyme, and more research is needed to elucidate the impact of mixing during bottling methods on micronutrients and other bioactive compounds in DHM. Additional research is also warranted to test larger pool volumes and stainless steel pooling containers.

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CHAPTER VI

EPILOGUE

Marie Curie once said, “the way of progress is neither swift nor easy.” The same can be said about a dissertation.

Conclusion

We observed that fat was affected by several steps in donor human milk (DHM) processing, including decanting, pooling, and mixing during bottling, as well the duration between pooling and bottling. Our results translate into the following suggestions for milk banks: The use of human milk analyzers to create targeted pools may be a useful tool for decreasing macronutrient variability in bottled DHM. For milk banks that do not create targeted pools, using a greater number of donors per pool may reduce both fat and protein variability. When processing milk frozen in a plastic storage bag, fat retention may be improved by using bag manipulation during decanting when thawing to a liquid state. When milk is pooled on one day and bottled on a subsequent day, more mixing is needed to reduce fat variability. By providing milk banks with practical strategies to mitigate nutrient variability, we may be able to improve growth outcomes in preterm infants fed DHM.

Challenges

Our observational study in Chapter III revealed numerous combinations of processing methods used in milk banks, indicating the need for controlled study designs to appropriately assess how each processing method impacts nutrient retention and distribution. However, a major challenge we encountered with our experimental studies (Chapter IV and V) was the inability to model all milk banking practices. For example, we did not test the impact of stainless steel pooling containers, large pool volumes spread across multiple containers, or thaw stages between ice and liquid.

In Chapter IV, we were unable to quantify the change in fat between fresh and post-thawed samples due to challenges that arose with the initial fat assay, creamatocrit, which was successful for measuring fat in all other parts of this dissertation. Aliquot volumes in this study were kept small in order to retain the maximum possible volume of milk to be used in this study, as well as a subsequent study (not part of this dissertation). Creamatocrit results for the fresh samples were within normal limits. After processing over half of the post-thawed samples, an unreasonable loss of fat was revealed. We hypothesized that vortexing small volumes of thawed milk (150 µL in a 1.5 mL microtube) was resulting in coalescence of milk fat globules, which were adhering to the container. The freeze/thaw cycle disrupts the milk fat globule membrane and allows fat to more readily aggregate (hence why this was not observed with fresh milk).[1] Fat is also more likely to form clumps when air is whipped into the milk during mixing, which was possible given the smaller volume of milk in a larger microtube.[1] Our revised study protocol increased aliquot volume from 150 µL to 1 mL. Even though coalescence was not observed during a preliminary investigation of measuring creamatocrit at a variety of temperatures, we also decided to warm the aliquots prior to analysis, since it has been shown to hinder fat aggregation.[2] Using the new method, fat content in the remaining samples fell within typical ranges, as did the results from previous samples that we retested. However, the retested milk had a longer storage duration and undergone an additional freeze/thaw cycle, which caused concerns about comparing samples after changes in fat structure. Additionally, a substantial number of samples (fresh and thawed) were below 3.0 g/dL, and creamatocrit has not been validated for milk below that level. All samples were then retested using the gravimetric method, which has been validated to measure a wider range of fat and is not sensitive to physical changes in fat that occur during frozen storage. The gravimetric method was not considered initially, as it takes much more time to complete than creamatocrit, and would not have been efficient in our study — specifically, study participants scheduled individual appointments to provide fresh samples, which were immediately tested for fat content. Although we retained aliquots of fresh milk, the volume was insufficient for the gravimetric assay. Comparisons between fresh and thawed milk would be dubious given the use of two different assays. However, we were able to compare post-thaw fat content using two different thaw stages and two different bag manipulation techniques, which allowed us to answer our primary study question regarding whether thaw stage or bag manipulation leads to improved fat content in decanted, previously frozen human milk.

In Chapter V, the untimely demise of our initial plate reader result in a limited number of samples measured for lysozyme, and measurements for protein and IgA spanning two different machines. The replacement plate reader was not equipped with temperature or mixing functions, so we conducted an exploratory analysis to determine if the second plate reader would be reliable for assays requiring those functions. Lysozyme is a temperature-dependent assay, and attempts to keep the plates warm were unsuccessful, with CVs for individual samples > 25%. The protein assay (BCA) did not require the plate reader to use any special features, and CVs for individual samples were < 5%. IgA is a mixing-dependent assay, and slightly more aggressive agitation prior to reading the plate seemed successful, with CVs for individual samples < 10%. However, initial data analysis revealed the plate reader to be a confounding variable, and we needed to stratify the analysis by plate reader, so we were unable to compare all mixing methods.

In addition to the significance of "mixing" revealed by our data in Chapters III and V, the theme of "mixing" had ironically emerged in the methods of Chapters III, IV and V. In Chapter IV, the vortexing of small-volume aliquots created fat aggregates in some samples, leading us to question our initial findings and revise a part of our methods. In Chapter V, the lack of a mixing function in the replacement plate reader necessitated a stratified analysis, and thus decreased analytical power from comparisons between fewer samples. Moreover, we speculated that insufficient mixing occurred at the milk bank-level was a likely explanation as to why two samples received for the Chapter III study had unreasonably high fat contents (repeated tests in our lab confirmed levels of fat in the DHM samples that were above physiological norms).

Implications for Future Research

Data presented in Chapter III highlight the negative relationship between variability and donors per pool, but only in the absence of target pooling — knowing the macronutrient composition of a donor's milk did not necessitate the use of additional donors to drive down fat and protein variability. The ability to create a consistent profile of nutrients in single-donor pools (which represented 17% of our samples) is especially useful for milk banks that produce specialty pools requiring milk from a limited subset of donors (e.g. vegetarian or nut-free diets). Future research is needed to investigate if our findings can be extrapolated to the vitamin and mineral content DHM.

Data presented in Chapter IV illustrated that squeezing and folding storage bags during decanting may be useful for retaining fat in milk thawed to a liquid state. The difference in fat retention between thaw stages was approaching significance and warrants further investigation with a larger sample. It is estimated that 97 participants would be needed to detect a difference in fat of 0.1 g/dL.[3] Future studies should also quantify pre– and post–thaw change in fat to gain a more in-depth perspective on how thaw stage and decanting method impact the retention of fat. Due to the minimum fat content limitations of creamatocrit, and the duration of time needed for gravimetric measurements, human milk analyzers may be a better method for measuring fat — some analyzer models are able to obtain accurate results in about a minute, using a 3 mL sample of milk.[4,5]

Data presented in Chapter V highlighted the importance of mixing after several hours of refrigerated storage, and future studies may be able to specify a dose-response relationship between duration of storage and duration of mixing needed to create bottles with an even distribution of nutrients. Our results indicate that larger pool volumes (close to those seen in milk banks) may be needed to observe differences, as well as pools spread across multiple containers (a common practice observed in the field). Also, future study analyses should investigate non-linear relationships between nutrients and pour order.

Closing Remarks

Original data presented in this dissertation provides insight to previously unexplored steps in DHM processing, and how those steps may affect the distribution and retention of macronutrients and bioactive compounds, although more research is needed to fully elucidate those relationships.

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APPENDIX A

MILK BANKING TERMS AND DEFINITIONS

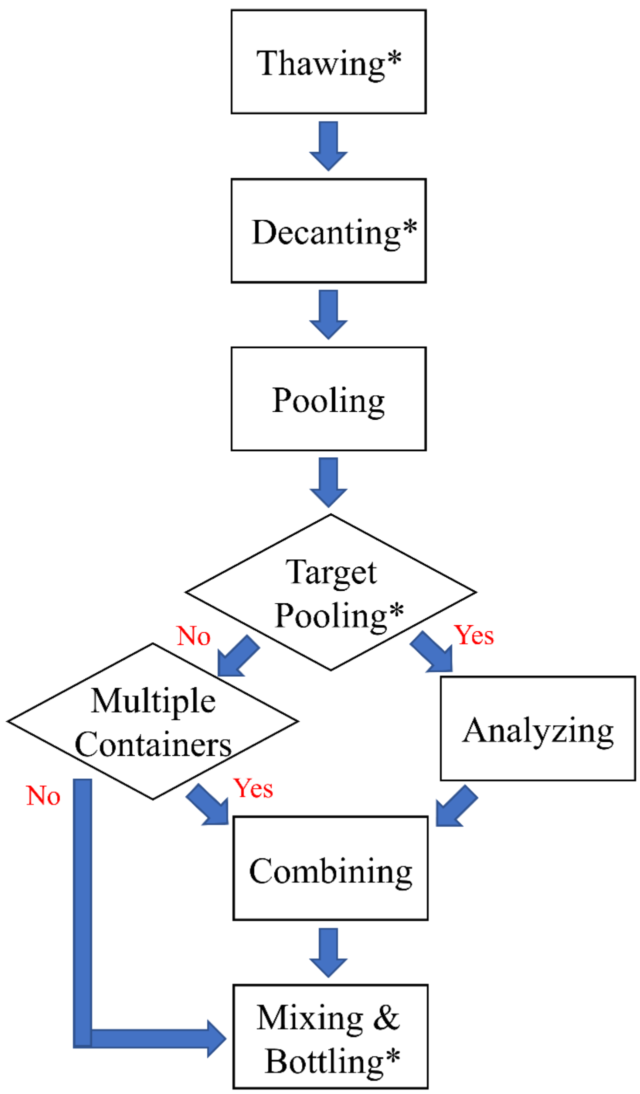
Term	**Definition**
Donor	A person who has been screened and approved to donate expressed human milk (HM) to a milk bank.
Single Sample	An individual container of HM, collected by an approved donor and frozen for donation. Typically 3–5 oz and most often contained in plastic storage bags designed for HM, but may sometimes be in plastic bottles.
Deposit	A unit of processing within a milk bank. Typically 50–80 oz total, a deposit contains multiple single samples donated by one person at one time. Deposits are often sorted by pump date so HM expressed at similar stages of lactation are grouped together. A donor can have more than one deposit.
Analyzing	The use of a human milk analyzer (HMA) to determine the macronutrients in HM. This may occur at one or more time points (e.g. for an individual donor after thawing and decanting, and/or for a collective pool that is ready to be bottled). This is not done in all milk banks.
Thawing	The process of HM going from a completely frozen state to a less frozen/more liquid state, where it is ready to be decanted from the original storage container. The degree and method of thaw varies by milk bank.
Decanting	The process of removing thawed HM from the original single sample container into one or more pooling containers to form a pool. An entire deposit is decanted at one time.
Bag Manipulation	A technique where physical force is exerted on a bagged single sample of HM during the decanting process, via squeezing and/or folding, in attempt to remove any remaining contents after the majority of HM has been poured out. This is not done in all milk banks.
Pooling Container	The vessel(s) for thawed HM. Usually glass or rigid plastic, but can also be stainless steel.
Pooling	The creation of a collection of decanted HM in one or more pooling container(s). A pool may be from one donor or multiple donors, and may be decanted into one container or multiple containers. Selection criteria for the pool varies by milk bank.
	Target Pool: A specific type of pool that is created using macronutrient data from an HMA to achieve a specific nutrient range. Not all milk banks create target pools.

Combining	The process of uniting multiple containers within a pool in order to create an equal distribution of nutrients across all containers. This is only done with multiple container pools. Techniques vary by milk bank.
	Pour Down Method: A method of combining where 6 containers are placed in a line, the first container is swirled for approximately 5 seconds and ≤ ⅓ of contents are poured into the second container, which is then swirled and ≤ ⅓ of contents poured into the third container, and so on. This is repeated 6 times. If there are less than 6 containers, then the number of pour downs is equal to the number of containers.
	Little Bit of This, Little Bit of That: A method of combining that is comparable to the pour down method, but not as regimented. Similar amounts of decanted HM are poured into an empty pooling container so it is ≤ ½ full. The pooling container is swirled for approximately 5 seconds, then poured into bottles. The process is repeated until the entire pool has been bottled.
Mixing During Bottling	The process of agitating the pooling container(s) while pouring HM into bottles, to ensure an even distribution of nutrients between the bottles in a pool. Techniques vary by milk bank.
	Manual: A method of mixing during bottling using intervals of hand swirling and hand pouring. Different combinations intervals were observed, ranging from swirling before each pour to swirling then pouring up to three bottles.
	Mechanical: A method of mixing during bottling using continuous stirring with a device (e.g. stand mixer, magnet, and/or oscillating plate) that occurs simultaneously during pouring (via dispensing pump or spout/spigot). The initial degree of agitation varies but decreases as the volume of HM decreases. The container (e.g. 5-gallon plastic bucket) is typically larger than what is used for manual mixing.
Batch	Bottles of HM from the same pool that were pasteurized in the same run of the pasteurizer.
Homogenizing	In the dairy industry, the term refers to the mechanical process of reducing the size of milk fat globules to prevent the milk from separating into fat and skim layers.
	With HM, the term is used to describe various degrees of mixing — from manually agitating a container (e.g. swirling a flask or inverting a microtube) to the use of an ultrasonicator (mechanized mixing via sound waves). Note: At the time of the environmental scan, no HMBANA banks were using ultrasonicators to process DHM.

APPENDIX B

FLOW CHART OF DONOR MILK PROCESSING

This flow chart provides a snapshot of donor milk processing. The impact of steps prior to thawing (e.g. storage and freezing) and after bottling (e.g. pasteurization) have been thoroughly investigated, and thus are not included. Key steps pertaining to this dissertation are indicated by an asterisk (*).



APPENDIX C

INTERVIEW GUIDE FOR ENVIRONMENTAL SCAN

This interview guide is an outline of questions used to direct visits with HMBANA milk banks during an environmental scan conducted between December 2016 and April 2018.

A. Thawing
    a. How are samples selected for thawing?
    b. What method is used (e.g. refrigerator, room temp, water bath)?
        i. How long does thawing take?
        ii. How is temperature monitored?
        iii. How are the samples stored (e.g. type of bin, open/closed bins, donors per bin)?
    c. What is the consistency of milk that is ready to be decanted (e.g. mostly frozen, some ice crystals, mostly liquid)?
B. Decanting
    a. Is this done under a hood? Are surgical towels used?
    b. How are the bags handled/inspected before decanting (e.g. shake, squeeze)?
        i. What happens to the milk from leaky bags (milk in the bag and milk that has leaked out)?
    c. How are the bags opened (e.g. scissors, how many at a time)?
    d. When are the samples filtered/strained?
        i. What type of filter/strainer is used (e.g. size, material)?
    e. What type of container is used (e.g. size, material)?
        i. How much volume is poured into one container?
        ii. How many donors per container (e.g. single donor pool)?
        iii. How many containers are needed?
    f. [Conduct visual inspection of discarded bags (e.g. volume, visible fat).]
C. Pooling
    a. How is the pool chosen (e.g. number of donors, size of donations, expiration date, macronutrient/total energy composition, volume, preterm)?
        i. Is the decision made pre– or post–thaw?
        ii. Are macronutrients analyzed?
        iii. Who has the ability/access to select the donor pool?
    b. What kind of container is used for the pool (e.g. size, material)?
        i. How much volume is in one container?
        ii. How many containers are needed?
    c. What is the method for combining (e.g. manual, homogenizer)?
        i. For mechanical combining: (e.g. magnet, wand)

                1. What is the speed/intensity?
                2. What is the duration?
            ii. For manual combining:
                1. How many swirls are done before each pour?
                2. How much is poured each time?
                3. How many times is this repeated (e.g. how many total pours)?
        d. Are macronutrients tested after pooling?
D. Bottling
    a. What are the containers/volumes (e.g. size, type, material, sealing)?
    b. Is this done under a hood? Are surgical towels used?
    c. How is milk mixed during bottling (e.g. continuous, manual)?
        i. For mechanical/continuous: (e.g. magnet, wand)
            1. What is the speed/intensity?
        ii. For manual mixing:
            1. How many swirls are done before each pour?
E. Pasteurizing
    a. What machine is used to pasteurize?
    b. What is the temperature/duration?
F. Total Processing
    a. How many ounces are typically processed per day?
        i. How many donors and how many pools does it take to create this volume?
        ii. How many runs of the pasteurization equipment does it take?
    b. What is the total processing time from thawing to pasteurizing?
G. Other questions
    a. Bags
        i. Are donors provided with bags?
        ii. What type of bags are most common?
        iii. What type of bags have the most problems (e.g. leaks, tears)?
        iv. What are the characteristics of an ideal bag?
    b. Analyzer
        i. When is milk tested for macronutrients?
        ii. What equipment is used?
        iii. Who performs these tests?
    c. Product loss
        i. What percent of all samples is lost?
        ii. At which step does most loss occur?
        iii. What are the reasons for loss (e.g. spill, contamination)?
        iv. When is milk tested for microorganisms?
    d. Training
        i. What kind of training is given to employees and volunteers? How long does it take?

APPENDIX D

MATRIX OF PRACTICES OBSERVED AT MILK BANKS

This matrix is a summary of practices observed at HMBANA milk banks during an environmental scan conducted between December 2016 and April 2018.

Milk Bank	**Thawing**	**Thaw Stage**	**Decanting**	**Target Pooling**	**Pooling Container**	**Mixing During Bottling**
1	Refrigerator and room temperature	Some ice crystals	No	No	Glass	Manual
2	Room temperature	Some ice crystals	Not typically	No	Glass	Manual
3	Refrigerator and room temperature	Some ice crystals	Not typically	Yes	Plastic	Manual
4	Refrigerator and room temperature	Mostly liquid	No	No	Glass	Manual
5	Room temperature	50/50 ice/liquid	Yes	No	Plastic	Mechanical
6	Refrigerator (water bath when necessary)	Mostly ice	Yes	Yes	Plastic	Mechanical
7	Room temperature	Some ice crystals	Yes	Yes	Plastic	Manual
8	Refrigerator	Completely liquid	No	Yes	Glass	Manual
9	Refrigerator and room temperature	Some ice crystals	Yes	No	Plastic	Mechanical